	L #	Hits	Search Text	DBs	Time Stamp
1	L1	286	ikk$2	USPAT; US-PGPUB	2003/03/24 15:29
2	L2	31634	kinase$1	USPAT; US-PGPUB	2003/03/24 15:29
3	L3	117	1 same 2	USPAT; US-PGPUB	2003/03/24 15:29
4	L4	54	1 same (human or murine or mouse)	USPAT; US-PGPUB	2003/03/24 15:29
5	L5	41	3 and 4	USPAT; US-PGPUB	2003/03/24 15:30

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TITLE: Anti-inflammatory compounds and uses thereof

PUBLICATION-DATE: March 20, 2003

INVENTOR-INFORMATION:

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RELATED-US-APPL-DATA:

non-provisional-of-provisional 60201261 20000502 US

US-CL-CURRENT: 514/15,514/16 ,514/17 ,530/326 ,530/327 ,530/328 ,530/329

ABSTRACT:

The present invention provides anti-inflammatory compounds, pharmaceutical compositions thereof, and methods of use thereof for treating inflammatory disorders. The present invention also provides methods of identifying anti-inflammatory compounds and methods of inhibiting NF-.kappa.B-dependent target gene expression in a cell.

RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application Serial No. 60/201,261 filed May 2, 2000 and to U.S. patent application Ser. No. 09/643,260 filed Aug. 22, 2000, the entire contents of each of which are incorporated herein by reference.

---------- KWIC ---------

Summary of Invention Paragraph - BSTX:

[0004] NF-.kappa.B is a transcription factor which mediates extracellular

signals responsible for induction of genes involved in pro-inflammatory responses (Baltimore et al., (1998) U.S. Pat. No. 5,804,374). NF-.kappa.B is anchored in the cytoplasm of most non-stimulated cells by a non-covalent interaction with one of several inhibitory proteins known as I.kappa.Bs (May & Ghosh, (1997) Semin. Cancer. Biol. 8, 63-73; May & Ghosh, (1998) Immunol. Today 19, 80-88; Ghosh et al., (1998) Annu. Rev. Immunol. 16, 225-260). Cellular stimuli associated with pro-inflammatory responses such as TNF.alpha., activate **kinases,** which in turn activate NF-.kappa.B by phosphorylating I.kappa.Bs. The **kinases** that phosphorylate I.kappa.Bs are called I.kappa.B **kinases (IKKs)**.

Summary of Invention Paragraph - BSTX:

[0006] The identification and characterization of **kinases** that phosphorylate I.kappa.Bs has led to a better understanding of signaling pathways involving NF-.kappa.B activation. Several different subtypes of **IKK** have been identified thus far. IKK.alpha. was initially identified as an I.kappa.B **kinase** induced by TNF.alpha. stimulation in HeLa cells (DiDonato et al., (1997) Nature 388, 548-554). Another I.kappa.B **kinase** homologous to IKK.alpha. was identified, termed IKK.beta. and determined to be the major I.kappa.B **kinase** induced following TNF.alpha. stimulation (Takeda et al., (1999) Science 284, 313-316; Hu et al., (1999) Science 284, 316-320; Li et al., (1999) Science 284, 321-325; Pot et al., (2000) U.S. Pat. No. 6,030,834; Woronicz & Goeddel (1999) U.S. Pat. No. 5,939,302). IKK.alpha. and IKK.beta. have an overall homology of 52% and a 65% homology in the **kinase** domain (Zandi et al., (1997) Cell 91, 243-252).

Summary of Invention Paragraph - BSTX:

[0007] I.kappa.B protein **kinases (IKKs)** phosphorylate I.kappa.Bs at specific serine residues. For example, they specifically phosphorylate serines 32 and 36 of I.kappa.B.alpha. (Traenckner et al., (1995) EMBO J. 14, 2876-2883; DiDonato et al., (1996) Mol. Cell. Biol. 16, 1295-1304). Phosphorylation of both sites is required to efficiently target I.kappa.B.alpha. for degradation. Furthermore, activation of IKK.alpha. and IKK.beta. is usually in response to NF-.kappa.B activating agents and mutant IKK.alpha. and IKK.beta., which are catalytically inactive, can be used to block NF-.kappa.B stimulation by cytokines such as TNF.alpha. and IL-1 (Rgnier et al., (1997) Cell 90, 373-383; Delhase et al., (1999) Science 284, 309-313). I.kappa.B protein **kinases** are therefore essential in the regulation of NF-.kappa.B activation processes.

Summary of Invention Paragraph - BSTX:

[0008] IKK.alpha. and IKK.beta. have distinct structural motifs including an amino terminal serine-threonine **kinase** domain separated from a carboxyl proximal helix-loop-helix (H-L-H) domain by a leucine zipper domain. These structural characteristics are unlike other **kinases,** and the non-catalytic domains are thought to be involved in protein-protein interactions. Proteins which bind to **IKKs** may therefore be capable of regulating the activity of

NF-.kappa.B (Marcu et al., (1999) U.S. Pat. No. 5,972,655) and potentially regulating downstream events such as induction of NF-.kappa.B. For instance, NEMO (NF-.kappa.B Essential Modulator) is a protein which has been identified to bind to **IKKs** and facilitate **kinase** activity (Yamaoke et al., (1998) Cell 93, 1231-1240; Rothwarf et al., (1998) Nature 395, 287-300; Mercurio et al., (1999) Mol. Cell. Biol. 19, 1526-1538; Haraj & Sun, (1999) J. Biol. Chem. 274, 22911-22914; Jin & Jeang, (1999) J. Biomed. Sci. 6, 115-120).

Brief Description of Drawings Paragraph - DRTX:

[0027] FIG. 4 depicts results from experiments indicating that association of NEMO with IKK.beta. and IKK.alpha. reveals the NEMO binding domain (NBD) to be six COOH-terminal amino acids. (A) COS cells transiently transfected with vector alone, FLAG-tagged IKK.alpha. or IKK.beta. (1 .mu.g/well) or xpress-tagged NEMO (1 .mu.g/well) to a total DNA concentration of 2 .mu.g/well as indicated. Following lysis, immunoprecipitations (IP) were performed using anti-FLAG (M2) and the contents of precipitates visualized by immunoblotting (IB) with either anti-FLAG (M2) or anti-xpress. A portion of pre-IP lysate was immunoblotted with anti-xpress to ensure equivalent levels of NEMO expression in transfected cells. (B) Wild-type IKK.alpha. and IKK.alpha.-(1-737) were expressed and labeled (input) and used for GST pull-down using GST or GST-NEMO. (C) Full length cDNA encoding **human IKKi** was obtained by RT-PCR from HeLa cell mRNA using the Expand.TM. Long Template PCR System (Boehringer Mannheim), the forward primer (5'-CTAGTCGAATTCACCATGCAGAGCACAGCCAATTAC) (SEQ ID NO: 22) and
the reverse primer (3'-CTAGTCTCTAGATTAGACATCAGGAGGTGCTGG) (SEQ ID NO: 23) and
cloned into the EcoRI and XhaI sites of pcDNA-3. GST pull-down analysis was performed using [.sup.35S]-methionine-labeled IKK.alpha., IKK.beta. and **IKKi**. (D) A deletion mutant of IKK.beta. lacking the NBD (del.NBD) was [.sup.35S]-methionine-labeled (input) and used for GST pull down analysis. (E) A Fauchere-Pliska hydrophobicity plot of the COOH-terminus (N721-S756) of **human** IKK.beta. was generated using MacVector.quadrature. (version 6.5.3) software. The NBD (L737-L742) is boxed. (F) COS cells were transfected for forty-eight hours with a total of 2 .mu.g DNA/well of either vector alone, vector plus NEMO-FLAG or NEMO-FLAG plus xpress-tagged versions of IKK.beta.-(1-744) containing point mutations within the NBD as indicated. Following lysis and immunoprecipitation using anti-FLAG (M2), immunoblot analysis was performed with either anti-FLAG or anti-xpress. The level of expressed protein in pre-IP lysate was determined by immunoblotting with anti-xpress (lower panel). (G) HeLa cells were transiently transfected for forty-eight hours with the indicated constructs together with pBIIX-luciferase and NF.kappa.B activity in lysate was measured by luciferase assay.

Detail Description Paragraph - DETX:

[0048] As used herein, the term "I.kappa.B-**kinase**" or "I.kappa.B protein **kinase**" or "I.kappa.B-**kinase** complex" or "I.kappa.B protein **kinase** complex" or "**IKK**" refers to a **kinase** that phosphorylates I.kappa.Bs.

Detail Description Paragraph - DETX:

[0050] As used herein, the term "NEMO" (NF-.kappa.B Essential Modulator), "IKK.gamma." or "**IKKAP**" refers to the protein which binds to **IKKs** and facilitates **kinase** activity.

Detail Description Paragraph - DETX:

[0180] These results demonstrate that basal auto-phosphorylation and **kinase** activity of IKK.beta. is dependent on the ability of NEMO to associate with the **kinase**. One explanation for these observations may be that NEMO recruits a phosphatase to the **IKK**-complex that regulates basal IKK.beta. function by targeting the serine-rich region of the COOH-terminus. Inability to bind NEMO therefore prevents phosphatase recruitment and causes increased phosphorylation within this region.

Detail Description Paragraph - DETX:

[0186] Further evidence that this short COOH-terminal sequence constitutes the NEMO-interaction domain of the **IKKs** was obtained when we tested the ability of the recently described **IKK**-related **kinase IKKi** (Shimada et al., (1999) Int. Immunol. 11, 1357-1362) to interact with NEMO. Sequence comparison with IKK.alpha. and IKK.beta. (Shimada et al., (1999) Int. Immunol. 11, 1357-1362; Woronicz et al., (1997) Science 278, 866-869; Zandi et al., (1997) Cell 91, 243-52; Mercurio et al., (1997) Science 278, 860-866; DiDonato et al., (1997) Nature 388, 548-554; Rgnier et al., (1997) Cell 90, 373-383) reveals that **IKKi** does not contain the .alpha.2-region in its COOH-terminus (Shimada et al., (1999) Int. Immunol. 11, 1357-1362) and consistent with this being the NEMO binding domain we found that **IKKi** does not interact with GST-NEMO in pull down assays (FIG. 4C). This finding indicates that NEMO is not required for the functional activity of **IKKi** and this is supported by the inability of **IKKi** to respond to signals induced by either TNF.alpha. or IL-1.beta. (Shimada et al., (1999) Int. Immunol. 11, 1357-1362).

Detail Description Paragraph - DETX:

[0194] The importance of the present invention can be viewed on two levels. First, Applicants have identified the structural requirements for the association of NEMO with the **IKKs** and found that association with IKK.beta. is dependent on three amino acids (D738, W739 and W741) within the NBD. Furthermore, NEMO not only functions in the activation of IKK.beta. but it also has a critical role in suppressing the intrinsic, basal activity of the **IKK** complex. The second level of importance is the obvious clinical use for drugs targeting the NBD. Applicants have demonstrated that a cell-permeable peptide encompassing the NBD is able to not only inhibit TNF.alpha.-induced NF-.kappa.B activation but also reduce expression of E-selectin, an NF-.kappa.B-dependent target gene, in primary **human** endothelial cells. The NBD is only six amino acids long, and therefore it is within the ability of one skilled in the art to design peptido-mimetic compounds that disrupt the core

IKK complex. Since the effect of disrupting the complex is to increase the basal activity of the **IKK,** treatment with an NBD-targeting compound can avoid issues of toxicity, e.g., due to hepatocyte apoptosis, that might arise from administering drugs that completely abolish the activity of NF-.kappa.B. Hence, identification of the NBD is a means for the development of novel anti-inflammatory drugs that prevent activating signals from reaching the **IKK** complex, yet maintain a low level of NF-.kappa.B activity and avoid potential toxic side-effects.

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TITLE: Composition and method for reconstituting IkB kinase in yeast and methods of using same

PUBLICATION-DATE: March 20, 2003

INVENTOR-INFORMATION:

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US-CL-CURRENT: 435/69.1,435/194 ,435/254.2 ,435/320.1 ,435/34

ABSTRACT:

The invention provides a means for reconstituting I.kappa.B **kinase** in yeast in order to study the structure and regulation of **IKK** and to produce pharmacological therapies to block **IKK**. This invention can be used to express an **IKK** complex that is biochemically identical to **IKK** isolated from native cells and in coupled in vitro **kinase** assays to screen for its upstream regulators. The **IKK** expressed by reconstituting the yeast can be used to screen for unknown substrates and for pharmacological therapies that block its activity. The invention could also be used to screen for compounds that enhance its activity. The **IKK** may also be used as a source of material for crystallization and X-ray diffraction analysis.

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 60/269,499, filed Feb. 16, 2001, the entire disclosure of which is hereby incorporated by reference in its entirety for all purposes.

---------- KWIC ---------

Abstract Paragraph - ABTX:

The invention provides a means for reconstituting I.kappa.B **kinase** in yeast in order to study the structure and regulation of **IKK** and to produce pharmacological therapies to block **IKK**. This invention can be used to express an **IKK** complex that is biochemically identical to **IKK** isolated from native cells and in coupled in vitro **kinase** assays to screen for its upstream regulators. The **IKK** expressed by reconstituting the yeast can be used to screen for unknown substrates and for pharmacological therapies that block its activity. The invention could also be used to screen for compounds that enhance its activity. The **IKK** may also be used as a source of material for crystallization and X-ray diffraction analysis.

Summary of Invention Paragraph - BSTX:

[0002] The present invention relates to molecular biology and biochemistry and, in particular, to reconstituting I.kappa.B **kinase ("IKK")** in yeast and using **IKK** expressed in yeast.

Summary of Invention Paragraph - BSTX:

[0003] A. Cellular Function of **IKK Kinase**

Summary of Invention Paragraph - BSTX:

[0010] A diverse array of signals traverse multiple signaling pathways to stimulate NF-.kappa.B. All the signaling pathways, with the exception of ultraviolet radiation (15), converge at a specific critical regulatory point: the phosphorylation of amino terminal serines on I.kappa.B. This phosphorylation is catalyzed by a large **kinase** complex, I.kappa.B **kinase ("IKK")** (8, 20, 44). Because phosphorylation of I.kappa.B.alpha. by **IKK** is the key step in activation of NF-.kappa.B, understanding the structure and regulation of the complex is critical and, as discussed below, could be used to develop therapies to treat a variety of inflammatory and autoimmune diseases.

Summary of Invention Paragraph - BSTX:

[0012] Some aspects of the structure of **IKK** are known in the art (43). In 1997, a research group, including one of the inventors of the present invention, isolated and sequenced **IKK**. (See U.S. Pat. No. 6,242,253). **IKK** is composed of two homologous **kinase** subunits IKK.alpha. and IKK.beta. (85 kD and 87 kD respectively) and a 52 kD regulatory subunit IKK.gamma. (8, 42, 44). The .alpha. and .beta. subunits are associated with each other via their leucine zippers (42). It is believed that an .alpha.-helical regions towards the N-terminus of IKK.gamma. interacts with six amino acids at the C-terminus of IKK.alpha. and IKK.beta. (19). IKK.gamma. is required for activation of **IKK** in response to TNF and other stimuli (27). Experiments show that interrupting this interaction leads to a higher basal **IKK** activity but prevents stimulation of **IKK** by TNF.alpha. (19). Recombinant iKK.gamma. forms dimers

and trimers (27), and it is possible that IKK.gamma. mediates formation of the large **IKK** complex.

Summary of Invention Paragraph - BSTX:

[0020] A popular method in signal transduction research is to use mammalian cell culture, such as HeLa cells or **mouse** embryonic fibroblasts, to overexpress a wild type or dominant negative protein in and to test for any effect on **IKK** activity. Cell culture can be used to show if a protein at least has the potential to be involved in a given network, but it has inherent problems. First, it is difficult to determine if the overexpressed protein acts directly or indirectly. Second, when an enzyme or regulatory protein is expressed at higher than normal levels, it may associate with proteins and networks where it may not normally localize. As a result, the protein may act non-specifically and yield misleading results.

Summary of Invention Paragraph - BSTX:

[0023] Transforming yeast cells to express **IKK** overcomes these difficulties inherent in bacteria, Sf9, and mammalian cell culture. Yet there are very few endogenous **kinases** that have been identified in yeast resulting in a very low background of endogenous activity. Because yeast are eukaryotic, they can produce native and functional enzyme. One can also transform a given yeast cell with multiple plasmids, each plasmid with a different selection marker and the gene for a different subunit of **IKK**. With yeast, it is also possible to isolate strains that consistently co-express multiple subunits of **IKK**.

Summary of Invention Paragraph - BSTX:

[0029] In yet another aspect of the invention, a mechanism for the regulation of the **IKK** complex is disclosed wherein IKK.gamma. regulates the autophosphorylation of the T loop residues in the **kinases** domain of IKK.beta.. When the T loop residues are phosphorylated, the **kinase** is active. This phosphorylation is required for activation of the **IKK** complex. In the inactive state, the T loop residues are not phosphorylated while the .gamma.BD serine(s) are phosphorylated, and the phosphorylation of the serine(s) prevents IKK.gamma. from facilitating self-activation. The activation of the complex requires dephosphorylation of these .gamma.BD serines, which then allows IKK.gamma. to facilitate autophosphorylation of IKK.beta. in the T loop.

Summary of Invention Paragraph - BSTX:

[0030] In another aspect, the present invention pertains to using the **IKK** expressed in yeast to develop drug and diagnostic therapies. The present invention can be used in coupled in vitro **kinase** assays to screen for its upstream regulators and can also be used in assays to screen for unknown substrates. It can be used to screen for pharmacological therapies to block its activity; likewise it can be used to screen for compounds that enhance its

activity. **IKK** expressed in yeast may also be a good source of material for crystallization and X-ray diffraction analysis.

Brief Description of Drawings Paragraph - DRTX:

[0032] FIG. 1. Western Blot Analysis of **Human IKK** Expressed in Yeast

Brief Description of Drawings Paragraph - DRTX:

[0035] FIG. 2. **Human IKK** Expressed in Yeast Forms a Large Complex Similar to **IKK** from HeLa Cells

Brief Description of Drawings Paragraph - DRTX:

[0037] Yeast expressing **human** IKK.alpha., IKK.beta., and IKK.gamma. were lysed, and the 65,000 g supernatant was applied to the superose 6 gel filtration column and chromatographed. IKK.alpha..beta..gamma. activity eluted as a large complex (similar to **IKK** from HeLa cells). **IKK** activity from .alpha..beta..gamma.-expressing yeast was also seen in smaller complexes; most likely these complexes contain the catalytic subunits as dimers without IKK.gamma.. The activity from yeast expressing IKK.beta. only elutes as a small 158-230 kD complex.

Brief Description of Drawings Paragraph - DRTX:

[0044] Regulatory serines (177 and 181) in the **kinase** domain (T loop) and putative regulatory serines (740 and 750) in the gamma binding domain (.gamma.BD) of IKK.beta. were mutated to alanines in order to prevent any phosphorylation and/or to glutamic acids in order to mimic the charge in the phosphorylated state. These mutated forms of IKK.beta. were expressed in yeast with and without IKK.gamma., and the complexes were partially purified from the lysates by superose 6 gel filtration. The **kinase** activity of the **IKK** complexes towards GST-I.kappa.B.alpha. was assessed as in FIG. 3.

Brief Description of Drawings Paragraph - DRTX:

[0045] Lane 1 shows the activity of IKK.beta. alone. Lane 2 shows that IKK.beta. reconstituted with IKK.gamma. had higher activity than IKK.beta. alone, indicating that IKK.gamma. facilitates self-activation of **IKK**. In lane 3, the T loop regulatory serines is mutated to glutamic acids to mimic phosphorylated state results in high activity which is not further activated by co-expression with IKK.gamma. (lane 4) indicating that the regulatory role of IKK.gamma. is upstream to the phosphorylation of the T loop serines. In lane 5, the T loop serines are mutated to alanines and completely inactived the **kinases**. As seen in lane 6, this mutated IKK.beta. T loop AA is not activated at all by the presence of IKK.gamma.. When the serine residues in the gamma binding domain are mutated to glutamic acid, IKK.beta. still has a low level

of **kinase** activity (see lane 7), similar to wild-type IKK.beta. (see lane 1).

Detail Description Paragraph - DETX:

[0052] As used herein, the term "isolated," when used in reference to an IKB **kinase** complex or to an **IKK** subunit of the invention, means that the protein complex or subunit is relatively free from contaminating lipids, proteins, nucleic acids or other cellular material normally associated with an **IKK** in a cell.

Detail Description Paragraph - DETX:

[0054] The **IKK** subunits have binding sites with high affinity for specific signaling molecules. By measuring the biological activity of the reconstituted **kinase,** the functional equivalence can be determined. Biological activity is measured using assays specifically designed for measuring the activity of the native **IKK,** including the assays described in the present invention. In addition, antibodies raised against the biologically active native **IKK** protein can be tested for their ability to bind to the functionally equivalent variant. Binding indicates that the protein has a conformation similar to the native **IKK**.

Detail Description Paragraph - DETX:

[0055] The disclosed method for reconstituting **IKK kinase** involves the coexpression in yeast cells of genes encoding all three subunits of the **IKK kinase** complex, IKK.alpha., IKK.beta. and IKK.gamma.. The subunits can be introduced in the yeast strain on a stable plasmid (e.g. pESC), or it can be integrated into the yeast chromosome using standard techniques (46).

Detail Description Paragraph - DETX:

[0059] The preferred method for determining the level of intracellular **IKK** phosphorylation is a colony Western blot using replica plates. It will be recognized that, although particularly convenient, the colony Western blot is but one example of many conventional assays which could be employed to determine levels of intracellular **IKK kinase** activity. In the colony Western blot procedure, cDNA library transformants are initially plated on media which do not contain an inducer of the promoter which drives expression of the CDNA insert. For example, if the GAL1 promoter is used to drive expression of the cDNA insert, the cDNA library transformants are initially plated on a medium containing 2% glucose. On this growth medium, cells containing the CDNA expression will grow, but the encoded cDNA product is not expressed. In order to express the encoded cDNA, the cDNA library transformants must be plated on a medium containing galactose. As another example, in the present invention, IKK.alpha. and IKK.beta. were subcloned into pESC ura and pESC trp vectors in which the galactose promoter region was replaced with the met promoter from the leu(met) vector. In these plasmids, the presence of methionine represses

expression of **IKK**, but expression is induced by removal of the methionine.

Detail Description Paragraph - DETX:

[0061] Following overnight incubation, the replica filters are removed from the growth medium plates, and the colonies are lysed in situ by soaking the replica filters in a lysis solution for a period of time sufficient to lyse cellular membranes. A number of different solutions are used for lysing cellular membranes, the formula described in the examples below are just one of many that could be used. The replica filters are then probed with monoclonal antibodies directed against the IKK.alpha., IKK.beta., and IKK.gamma. subunits as well as the tag HA. Colonies which exhibit elevated **IKK kinase** activity on the replica filter which had been incubated overnight on a growth medium containing a compound which induces expression of the cDNA insert linked to the inducible promoter.

Detail Description Paragraph - DETX:

[0062] The method of the present invention is not limited to the reconstitution of **IKK** complex in yeast. Rather, the method can be modified for use reconstituting a number of different **kinases** and proteins--especially those composed of multiple subunits.

Detail Description Paragraph - DETX:

[0070] **Human IKK** activity from non-stimulated or TNF-stimulated HeLa cells was compared to yhIKK (.beta., .beta..gamma., and .alpha..beta..gamma.). yhIKK (.alpha..beta..gamma. or .beta..gamma.) activity was found to be slightly higher than the activity from TNF-stimulated HeLa cells. **Human** IKK.beta. expressed in yeast had a level of activity similar to TNF-stimulated HeLa cells. In these experiments **IKK** levels were similar in the yeast and HeLa extracts as assessed by Western blot as shown in FIG. 3. The heterologously expressed **IKK** comples is substantially homologous to **IKK** isolated from mammalian cells.

Detail Description Paragraph - DETX:

[0071] This example provides a method for identifying mechanisms for the regulation of the **IKK** complex. For example, by mutating to alanines (to prevent any phosphorylation) and/or to glutamic acids (to mimic the charge in the phosphorylated state) regulatory serines (177 and 181) in the **kinase** domain (T loop) and putative regulatory serines (740 and 750) in the gamma binding domain (.gamma.BD) of IKK.beta. and then expressing and partially purifying these mutated forms of IKK.beta. in yeast with and without IKK.gamma., the following mechanism for the regulation of the **IKK** complex is suggested: IKK.gamma. regulates the autophosphorylation of the T loop residues in the **kinases** domain of IKK.beta.. This phosphorylation is required for activation of the **IKK** complex. In the inactive state, the T loop residues are not

phosphorylated while the .gamma.BD serine(s) are phosphorylated, and the phosphorylation of the serine(s) prevents IKK.gamma. from facilitating self-activation. The activation of the complex requires dephosphorylation of these .gamma.BD serines, which then allows IKK.gamma. to facilitate autophosphorylation of IKK.beta. in the T loop. When the T loop residues are phosphorylated, the **kinase** is active.

Detail Description Paragraph - DETX:

[0072] In this experiment, the regulatory serines (177 and 181) in the T loop and putative regulatory serines (740 and 750) in the gamma binding domain .gamma.BD of IKK.beta. were mutated to alanines in order to prevent phosphorylation and/or to glutamic acids in order to mimic the charge in the phosphorylated state. These mutated forms of IKK.beta. were expressed in yeast with and without IKK.gamma.. The complexes were partially purified from the lysates by superose 6 gel filtration. The **kinase** activity of the **IKK** complexes towards GST-I.kappa.B.alpha. was assessed as in FIG. 3.

Detail Description Paragraph - DETX:

[0074] Mutation of the T loop serines to alanines makes the **kinase** completely inactive (lane 5), and this mutated IKK.beta. T loop AA cannot be activated at all by the presence of IKK.gamma. (lane 6). When the serine residues in the gamma binding domain are mutated to glutamic acid, IKK.beta. still has a low level of **kinase** activity (lane 7), similar to wild-type IKK.beta. (lane 1). However, reconstitution with IKK.gamma. does not allow IKK.beta..gamma.BDEE to become activated (lane 8). This result indicates that the analog of phosphorylated serines in the gamma binding domain prevents IKK.gamma. from facilitating self-activation of **IKK** and suggests that phosphorylation of these amino acids is a mechanism to maintain **IKK** in an inactive state.

Detail Description Paragraph - DETX:

[0078] In another embodiment, the present invention can be used to develop a method for assaying **IKK** activity in situ in yeast. The present invention provides two methods for using the present invention to screen for upstream regulators of **IKK**. First, because the **IKK** complex reconstituted in yeast is only partially active, the protein extracts from cytokine stimulated mammalian cells can activate the **IKK** complex in coupled in vitro **kinase** assays. Such activity can be then purified biochemically to identify the protein components of it. Second, as described below in EXAMPLE IV, a system can be established to isolate potential negative regulators in situ in yeast. The in situ system is based on determining the activity of **IKK** in yeast by assessing an antibody that recognizes only the phosphorylated form of I-.kappa.B.alpha.ser 32.

Detail Description Paragraph - DETX:

[0080] As indicated by these assays, I.kappa.B.alpha. is phosphorylated in

yeast by IKK.beta.:IKK.gamma. complex and the phosphorylation can be detected by phosphoantibodies to I.kappa.B.alpha.. I.kappa.B.alpha. was not phosphorylated in yeast that does not express **IKK** complex or expresses **kinase** defective IKK.beta.:IKK.gamma..

Detail Description Paragraph - DETX:

[0084] Finally, as a positive control to test whether the **IKK** is negatively regulated in the yeast cell, PP2A, all three subunits under antibiotic selection markers, are transformed into the yeast with a galactose promoter, allowing the phosphatase to be induced before the **kinase**. Induction of PP2A before **IKK** generates a yeast cell in which the **IKK** is present but not active. The resulting yeast have much less phosphorylation of I-.kappa.B.alpha.. For these assays, antibodies against PP2A are used in order to assess the expression of PP2A.

Detail Description Paragraph - DETX:

[0089] The invented composition can also be used to screen for pharmacological therapies to block its activity. This can be accomplished in two ways: first, libraries of small molecule compounds can be tested in in vitro **kinase** assays to inhibit or further activate the **IKK** complex made in yeast. For example, partially purified **IKK** complex from yeast can be incubated with a small molecule prior to testing its activity by in vitro **kinase** assay. Because this is a simple assay, a large number of compounds can be tested to determine if they inhibit or activate **IKK**. Second, the yeast system described above in EXAMPLE III, can be used to screen for small molecules that would inhibit or further activate the activity of **IKK** in situ (yeast).

Detail Description Paragraph - DETX:

[0110] 18. Makris, C., V. L. Godfrey, G. Krahn-Senftleben, T. Takahashi, J. L. Roberts, T. Schwarz, L. Feng, R. S. Johnson, and M. Karin 2000. Female mice heterozygous for **IKK** gamma/NEMO deficiencies develop a dermatopathy similar to the **human** X-linked disorder incontinentia pigmenti. Mol. Cell. 5:969-979.

Detail Description Paragraph - DETX:

[0112] 20. Mercurio, F., H. Zhu, B. W. Murray, A. Shevchenko, B. L. Bennett, J. Li, D. B. Young, M. Barbosa, M. Mann, A. Manning, and A. Rao 1997. **IKK-1 and IKK**-2: cytokine-activated IkappaB **kinases** essential for NF-kappaB activation. Science. 278:860-866.

Detail Description Paragraph - DETX:

[0119] 27. Rothwarf, D. M., E. Zandi, G. Natoli, and M. Karin 1998. **IKK**-gamma is an essential regulatory subunit of the IkB **kinase** complex. Nature.

395:297-300.

Detail Description Paragraph - DETX:

[0136] 44. Zandi, E., D. M. Rothwarf, M. Delhase, M. Hayakawa, and M. Karin 1997. The IkappaB **kinase** complex (**IKK**) contains two **kinase** subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. Cell. 91:243-252.

Detail Description Table CWU - DETL:

1TABLE 1 plasmids for expressing **IKK** in yeast Selection Subunit Plasmid name Tag(s) promoter marker of **IKK** Mutation Date pESC leu IKK.alpha. Myc gal leucine IKK.alpha. None July 1999 pESC his IKK.beta. HA gal histidine IKK.beta. None July 1999 pESC trp IKK.gamma. FLAG gal tryptophan IKK.gamma. None July 1999 6his pES86(+)IKK.gamma. FLAG ADH uracil IKK.gamma. None 15 Nov. 1999 6his leu(met) IKK.gamma. FL HA met leucine IKK.gamma. None 21 Apr. 2000 leu(met) IKK.gamma..DELTA.C300 HA met leucine IKK.gamma. C terminus 21 Apr. 2000 Deletion pESC his IKK.beta. .sub.KA HA gal histidine IKK.beta. **Kinase** 16 June 2000 defective PESC his IKK.beta..sub.EEM10 HA gal histidine IKK.beta. Constitutively 16 June 2000 active pESC ura (met) IKK.alpha. HA met uracil IKK.alpha. None 30 May 2000 pESC trp (met) IKK.beta. HA met tryptophan IKK.beta. None 10 July 2000 pESC trp (met) IKK.beta..sub.KA HA met tryptophan IKK.beta. **Kinase** 10 July 2000 defective pESC trp (met) IKK.beta..sub.EEM10 HA met tryptophan IKK.beta. Constitutively 10 July 2000 active pESC ura (met) IKK.alpha..sub.del NBD HA met uracil IKK.alpha. Remove NBD 6 Nov. 2000 pESC trp (met) IKK.beta..sub.del NBD HA met tryptophan IKK.beta. Remove NBD 6 Nov. 2000 leu (met) IKK.gamma.del HA met leucine IKK.gamma. Remove .gamma. 3 Jan. 2001 interaction domain

Claims Text - CLTX:

19. The method of claim 1.a, wherein said mammalian **IKK is human IKK**.

Claims Text - CLTX:

27. A heterologously expressed **IKK** complex, wherein said IKK.gamma. protein subunit regulates phosphorylation of serine residues in the activation of T loop **kinase** domain of **IKK** catalytic subunits.

Claims Text - CLTX:

32. A method for identifying upstream regulators of **IKK** complex, comprising the steps of: a. mutating the genes of one or more said **IKK** subunits; b. subcloning genes for **IKK** subunits into yeast expression vectors; c. transforming said yeast expression vectors into yeast; d. growing said yeast in a selective liquid media; e. controllably inducing the expression of said

IKK subunits by means of inducible promoters; f. lysing said yeast; g. extracting said **IKK** protein; h. purifying said **IKK** protein; and i. comparing **kinase** activity of said **IKK** protein with wild type **IKK**.

PGPUB-DOCUMENT-NUMBER: 20030045515

PGPUB-FILING-TYPE: new

DOCUMENT-IDENTIFIER: US 20030045515 A1

TITLE: Combination medicament for treatment of neoplastic diseases

PUBLICATION-DATE: March 6, 2003

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
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Vig	Holte		DK	
Hamberg, Karin Jexner				

APPL-NO: 10/ 151094

DATE FILED: May 21, 2002

RELATED-US-APPL-DATA:

non-provisional-of-provisional 60292928 20010524 US

US-CL-CURRENT: 514/210.2,514/217.04 ,514/353

ABSTRACT:

Pharmaceutical compositions comprising, as a first anti-neoplastic drug, cyanoguanidine IKK inhibitors, and in particular compounds of formula I 1

wherein

n is 0, 1 or 2;

each R independently represents halogen, trifluoromethyl, hydroxy, C.sub.1-4 alkyl, C.sub.1-4 alkoxy, C.sub.1-4 alkoxycarbonyl, nitro, sulfo, cyano, amino or carboxy groups; Q is a straight or branched, saturated or unsaturated C.sub.4-20 divalent hydrocarbon radical;

X is a bond, amino, O, S, carbonyl, carbonylamino, aminocarbonyl, oxycarbonyloxy, oxycarbonyl, carbonyloxy, aminocarbonyloxy, aminothiocarbonyloxy, oxycarbonylamino or oxythiocarbonylamino;

A is di-(C.sub.1-4 alkoxy)phospinoyloxy, C.sub.1-4 alkoxycarbonyl, C.sub.1-4 alkoxycarbonylamino, C.sub.3-12 carbocyclic ring or C.sub.3-12 heterocarbocyclic ring optionally substituted with one or more R.sub.1; R.sub.1 being independently selected from the group consisting of halogen,

trifluoromethyl, hydroxy, C.sub.1-4 alkyl, C.sub.1-4 alkoxy, C.sub.1-4 alkoxycarbonyl, nitro, cyano, amino, sulfo, carboxy, carboxamido, sulfamoyl or C.sub.1-4 hydroxyalkyl; in combination with a second anti-neoplastic drug are provided.

---------- KWIC ---------

Summary of Invention Paragraph - BSTX:

[0011] At the cellular level it is well recognised that Nuclear Factor .kappa.B (NF.kappa.B) plays a pivotal role in apoptosis and resistance to apoptosis. It is also described that an NF.kappa.B inhibitor, I.kappa.B, and an I.kappa.B **kinase** complex, **IKK**, control the level of activated NF.kappa.B [Levkau, 1, 227-233, 1999; Wang, Science, 274, 784-787, 196; Madrid, Molecular and Cellular Biology, 5, 1626-1638, 2000]. Accordingly, the NF.kappa.B-I.kappa.B-**IKK** system has been suggested as a target in the treatment of neoplastic diseases.

Summary of Invention Paragraph - BSTX:

[0015] It has surprisingly been found that certain cyanoguanidine compounds are capable of modulating the level of activated NF.kappa.B through inhibition of the I.kappa.B **kinase** complex (abbreviated **IKK** in the following), thereby preventing resistance to the apoptosis effected by other anti-neoplastic drugs and ionising radiation. Cyanoguanidine compounds are thus able to increase the effect of other anti-neoplastic treatments. Synergistic effects may therefore be obtained in the treatment of patients with neoplastic diseases by combining treatment with cyanoguanidine compounds with other types of anti-neoplastic treatment, e.g. treatment with chemotherapeutic agents, hormonal agents, biological response modifiers, angiogenesis inhibitors, differentiating agents and ionising radiation.

Summary of Invention Paragraph - BSTX:

[0063] NF.kappa.B is a member of the Rel family of transcription factors, which are ubiquitous in animal cells. Rel proteins can form dimers, the most common of which is designated NF.kappa.B. NF.kappa.B is a p50/p65 heterodimer which can activate transcription of genes containing the appropriate .kappa.B binding site. In non-stimulated cells, NF.kappa.B is maintained in the cytoplasm by an interaction with NF.kappa.B inhibiting proteins, the I.kappa.BS. In response to cell stimulation by e.g. anti-neoplastic drugs or ionising radiation an I.kappa.B **kinase** complex (**IKK**) is rapidly activated and phosporylates two serine residues in the NF.kappa.B binding domain of I.kappa.B. The phophorylated I.kappa.B is then degraded by a 26S proteasome whereas NF.kappa.B is spared from degradation and translocates into the nucleus [Wang, Science, 274, 784-787, 1996, Cusak, Cancer Research, 60, 2323-2330, 2000; Karin, Immunology, 12, 2000, 85-98]. NF.kappa.B is thus always present in the cell, but in an inactivated form in non-stimulated cells. After translocation into the nucleus NF.kappa.B induces inter alia the anti-apoptotic genes c-IAP1, c-IAP2, TRAF1, TRAF2, Bfl-1/A1, Bcl-X.sub.L and Mn-SOD [Platel, Oncogene, 19,

2000, 4159-4169], which bring about resistance in the cells to apoptosis. This effect is referred to as the anti-apoptotic effect of NF.kappa.B, and the effect may be quantified by measuring the expression of compounds encoded by any of said genes, by any suitable means known in the art, in the presence and absence of compounds modulating the level of activated NK.kappa.B. Any compound capable of reducing the transcription of said genes to a level less than 50%, e.g. less than 30%, such as less than 20% of the level in the absence of said compound is said to reduce the antiapoptotic effect of NK.kappa.B. Anti-neoplastic drugs and ionising radiation thus induce resistance in the cells to the treatments, which render them ineffective. Accordingly, activated NF.kappa.B is a key factor in induced resistance in e.g. cancer cells to anti-neoplastic drugs and/or to ionising radiation. This is further supported by the fact that constitutively activated NF.kappa.B is found in cells from resistant cancer tumours [Patel, Oncogene, 19, 4159-4169, 2000].

Summary of Invention Paragraph - BSTX:

[0067] The I.kappa.B **kinase** complex consist of three subunits, namely IKK.alpha., IKK.beta. and IKK.gamma., with a combined molecular weight of 900 kDa. IKK.alpha. and IKK.beta. both exhibit I.kappa.B **kinase** activity and phophorylate I.kappa.B, whereas IKK.gamma. is a regulatory subunit. IKK.alpha. is 85 kDa protein and IKK.beta. is a 87 kDa protein, and the two subunits show a large degree of homology. Whereas both IKK.alpha. and IKK.beta. are catalytically active, it has surprisingly been shown that only IKK.beta. is essential for **IKK** phosphorylation of I.kappa.B.

Summary of Invention Paragraph - BSTX:

[0086] In the systemic treatment using the present invention daily doses of from 0.001-200 mg per kilogram body weight, preferably from 0.002-50 mg/kg of mammal body weight, for example 0.003-10 mg/kg of a cyanoguanidine **IKK** inhibitor, e.g. a compound of formula I and a second anti-neoplastic drug are administered, typically corresponding to a daily dose for an adult **human** of from 0.2 to 1500 mg of each compound. In the topical treatment of dermatological disorders, ointments, creams or lotions containing from 0.1-750 mg/g, and preferably from 0.1-500 mg/g, of a cyanoguanidine **IKK** inhibitor, e.g. a compound of formula I and a second anti-neoplastic drug are administered. For topical use in ophthalmology ointments, drops or gels containing from 0.1-750 mg/g, and preferably from 0.1-500 mg/g, of a cyanoguanidine **IKK** inhibitor, e.g. a compound of formula I and a second anti-neoplastic drug are administered. The oral compositions are formulated, preferably as tablets, capsules, or drops, containing from 0.05-250 mg, preferably from 0.1-125 mg, of a cyanoguanidine **IKK** inhibitor, e.g. a compound of formula I and a second anti-neoplastic drug per dosage unit.

PGPUB-DOCUMENT-NUMBER: 20030040083

PGPUB-FILING-TYPE: new

DOCUMENT-IDENTIFIER: US 20030040083 A1

TITLE: Dual specificity antibodies and methods of making and using

PUBLICATION-DATE: February 27, 2003

INVENTOR-INFORMATION:

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Kaymakcalan, Zehra	Westboro	MA	US	

APPL-NO: 09/ 894550

DATE FILED: June 28, 2001

RELATED-US-APPL-DATA:

non-provisional-of-provisional 60215379 20000629 US

US-CL-CURRENT: 435/70.21,530/388.23

ABSTRACT:

Antibodies having dual specificity for two different but structurally related antigens are provided. The antibodies can be, for example, entirely human antibodies, recombinant antibodies, or monoclonal antibodies. Preferred antibodies have dual specificity for IL-1.alpha. and IL-1.beta. and neutralize IL-1.alpha. and IL-1.beta. activity in vitro and in vivo. An antibody of the invention can be a full-length antibody or an antigen-binding portion thereof. Methods of making and methods of using the antibodies of the invention are also provided. The antibodies, or antibody portions, of the invention are useful for detecting two different but structurally related antigens (e.g., IL-1.alpha. and IL-1.beta.) and for inhibiting the activity of the antigens, e.g., in a human subject suffering from a disorder in which IL-1.alpha. and/or IL-1.beta. activity is detrimental.

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims benefit of U.S. Provisional Application No. 60/215,379, filed Jun. 29, 2000.

---------- KWIC ---------

Summary of Invention Paragraph - BSTX:

[0129] The antibodies of the invention, or antigen binding portions thereof, may also be combined with agents, such as methotrexate, 6-MP, azathioprine sulphasalazine, mesalazine, olsalazine chloroquinine/hydroxychloroquine, pencillamine, aurothiomalate (intramuscular and oral), azathioprine, cochicine, corticosteroids (oral, inhaled and local injection), beta-2 adrenoreceptor agonists (salbutamol, terbutaline, salmeteral), xanthines (theophylline, aminophylline), cromoglycate, nedocromil, ketotifen, ipratropium and oxitropium, cyclosporin, FK506, rapamycin, mycophenolate mofetil, leflunomide, NSAIDs, for example, ibuprofen, corticosteroids such as prednisolone, phosphodiesterase inhibitors, adensosine agonists, antithrombotic agents, complement inhibitors, adrenergic agents, agents which interfere with signaling by proinflammatory cytokines such as TNF.alpha. or IL-1 (e.g. IRAK, NIK, **IKK** , p38 or MAP **kinase** inhibitors), IL-1.beta. converting enzyme inhibitors, TNF.alpha. converting enzyme (TACE) inhibitors, T-cell signalling inhibitors such as **kinase** inhibitors, metalloproteinase inhibitors, sulfasalazine, azathioprine, 6-mercaptopurines, angiotensin converting enzyme inhibitors, soluble cytokine receptors and derivatives thereof (e.g. soluble p55 or p75 TNF receptors and the derivatives p75TNFRIgG (Enbrel.TM. and p55TNFRIgG (Lenercept)), sIL-1RI, sIL-1RII, sIL-6R) and antiinflammatory cytokines (e.g. IL-4, IL-10, IL-11, IL-13 and TGF.beta.). Preferred combinations include methotrexate or leflunomide and in moderate or severe rheumatoid arthritis cases, cyclosporine.

Summary of Invention Paragraph - BSTX:

[0130] Non-limiting examples of therapeutic agents for inflammatory bowel disease with which an antibody, or antibody portion, of the invention can be combined include the following: budenoside; epidermal growth factor; corticosteroids; cyclosporin, sulfasalazine; aminosalicylates; 6-mercaptopurine; azathioprine; metronidazole; lipoxygenase inhibitors; mesalamine; olsalazine; balsalazide; antioxidants; thromboxane inhibitors; IL-1 receptor antagonists; anti-IL-1.beta. monoclonal antibodies; anti-IL-6 monoclonal antibodies; growth factors; elastase inhibitors; pyridinyl-imidazole compounds; antibodies to or antagonists of other **human** cytokines or growth factors, for example, TNF, LT, IL-2, IL-6, IL-7, IL-8, IL-12, IL-15, IL-16, IL-18, EMAP-II, GM-CSF, FGF, and PDGF. Antibodies of the invention can be combined with antibodies to cell surface molecules such as CD2, CD3, CD4, CD8, CD25, CD28, CD30, CD40, CD45, CD69, CD90 or their ligands. The antibodies of the invention, or antigen binding portions thereof, may also be combined with agents, such as methotrexate, cyclosporin, FK506, rapamycin, mycophenolate mofetil, leflunomide, NSAIDs, for example, ibuprofen, corticosteroids such as prednisolone, phosphodiesterase inhibitors, adenosine agonists, antithrombotic agents, complement inhibitors, adrenergic agents, agents which interfere with signalling by proinflammatory cytokines such as TNF.alpha. or IL-1 (e.g. IRAK, NIK, **IKK,** p38 or MAP **kinase** inhibitors), IL-1.beta. converting enzyme inhibitors, TNF.beta. converting enzyme inhibitors, T-cell signalling inhibitors such as **kinase** inhibitors, metalloproteinase inhibitors, sulfasalazine, azathioprine, 6-mercaptopurines, angiotensin converting enzyme

inhibitors, soluble cytokine receptors and derivatives thereof (e.g. soluble p55 or p75 TNF receptors, sIL-1RI, sIL-1RII, sIL-6R) and antiinflammatory cytokines (e.g. IL-4, IL-10, IL- 11, IL-13 and TGF.beta.).

Summary of Invention Paragraph - BSTX:

[0132] Non-limiting examples of therapeutic agents for multiple sclerosis with which an antibody, or antibody portion, of the invention can be combined include the following: corticosteroids; prednisolone; methylprednisolone; azathioprine; cyclophosphamide; cyclosporine; methotrexate; 4-aminopyridine; tizanidine; interferon-.beta.1a (Avonex; Biogen); interferon-.beta.1b (Betaseron; Chiron/Berlex); Copolymer 1 (Cop-1; Copaxone; Teva Pharmaceutical Industries, Inc.); hyperbaric oxygen; intravenous immunoglobulin; clabribine; antibodies to or antagonists of other **human** cytokines or growth factors, for example, TNF, LT, IL-2, IL-6, IL-7, IL-8, IL-12, IL-15, IL-16, IL-18, EMAP-II, GM-CSF, FGF, and PDGF. Antibodies of the invention, or antigen binding portions thereof, can be combined with antibodies to cell surface molecules such as CD2, CD3, CD4, CD8, CD25, CD28, CD30, CD40, CD45, CD69, CD80, CD86, CD90 or their ligands. The antibodies of the invention, or antigen binding portions thereof, may also be combined with agents, such as methotrexate, cyclosporine, FK506, rapamycin, mycophenolate mofetil, leflunomide, NSAIDs, for example, ibuprofen, COX-2 inhibitors, corticosteroids such as prednisolone, phosphodiesterase inhibitors, adensosine agonists, antithrombotic agents, complement inhibitors, adrenergic agents, agents which interfere with signalling by proinflammatory cytokines such as TNF.alpha. or IL-1 (e.g. IRAK, NIK, **IKK,** p38 or MAP **kinase** inhibitors), IL-1.beta. converting enzyme inhibitors, TACE inhibitors, T-cell signalling inhibitors such as **kinase** inhibitors, metalloproteinase inhibitors, sulfasalazine, azathioprine, 6-mercaptopurines, angiotensin converting enzyme inhibitors, soluble cytokine receptors and derivatives thereof (e.g. soluble p55 or p75 TNF receptors, sIL-1RI, sIL-1RII, sIL-6R) and antiinflammatory cytokines (e.g. IL-4, IL-10, IL-13 and TGF.beta.).

PGPUB-DOCUMENT-NUMBER: 20030036095

PGPUB-FILING-TYPE: new

DOCUMENT-IDENTIFIER: US 20030036095 A1

TITLE: Highly sensitive proteomic analysis methods, and kits and systems for practicing the same

PUBLICATION-DATE: February 20, 2003

INVENTOR-INFORMATION:

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APPL-NO: 09/ 960716

DATE FILED: September 21, 2001

RELATED-US-APPL-DATA:

non-provisional-of-provisional 60234527 20000922 US

US-CL-CURRENT: 435/7.1,427/2.11 ,435/7.9

ABSTRACT:

Methods of determining whether a sample includes one or more analytes, particularly proteinaceous analytes, of interest are provided. In the subject methods, an array of binding agents, where each binding agent includes an epitope binding domain of an antibody, is contacted with the sample. In many embodiments, contact occurs in the presence of a metal ion chelating polysaccharide, e.g., a pectin. Following contact, the presence of binding complexes on the array surface are detected and the resultant data is employed to determine whether the sample includes the one or more analytes of interest. Also provided are kits, systems and other compositions of matter for practicing the subject methods. The subject methods and compositions find use in a variety of applications, including proteomic applications such as protein expression analysis, e.g., differential protein expression profiling.

[0001] CROSS-REFERENCE TO RELATED APPLICATIONS

[0002] Pursuant to 35 U.S.C. .sctn.119(e), this application claims priority to the filing date of U.S. Provisional Patent Application Serial No. 60/234,527 filed Sep. 22, 2000, the disclosure of which is herein incorporated by reference.

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Detail Description Table CWU - DETL:

1 Antibody Table 14-3-3 e v.2 53BP2 v.2 ABP-280 ABR ACH ESTERASE ACH rec B ACTR/AIB1/RAC3 adaptin alpha adaptin beta ADAPTIN d v.3 Adaptin gamma AF6/p180 AFAP AIM-1 AKAP 149 AKAP 79 AKAP-KL ALDH alpha-/beta-SNAP AMPHIPHYSIN AMPK b v.2 Annexin II Annexin XI AP 180 v.2 ApoE APOLIPOPROTEIN App-BP1 ARF-3 B100 ARGINASE I ATAXIN-2 v.2 ATTRACTIN B CATENIN B NAP v.2 B56-alpha BAG-1/RAP46 BCL-2 Bcl-xl beta 1 Ca channel beta-Arrestin 1 BM28 v.2 BMXv.2 Bog bPIX BRAMP2/AMPHIPHYSIN 2 BRM BRUCE Btf c-cbl C-NAP1 Cadherin (5) Cadherin (E) Cadherin (P) CAF1 p150 CALCINEURIN Calnexin CALRETININ v.2 Calsequestrin CAM KIN KIN v.2 Casein K1e casein kin.IIb Casein **kinase** 1 Catenin (alpha) Cathepsin D Cathepsin L epsilon Caveolin 2 CDC 34 CDC 37 CDC27 hs CDC42GAP (Rac1) CDK 2 CDK4 CDK7 cGB-PDE v.2 CHD3 CHGB Chromogranin A/CGA CIP1 CLA-1 Clathrin HC CLIP-115 COL7A1 COMT Contactin v.2 COX-2 CPG16/DCAMKL1 CRIK CRP-1 Csk v.3 CUL-2 v.2 CUL-3 CYCLIN D3 Cypher1 DAP3 DARPP-32 v.2 DBP2 DBP2 DDX-1 DEK DEMATIN v.2 DFF45 DGKTHETA v.2 DHFR DLG-1 v.2 DLP1 DMPK DNA Polymerase DNA Polymerase e Doublecortin v.2 delta (catalytic) DSIF DYNAMIN 1 v.2 Dynamin II DYRK EB1 EBP5O v.2 EEA-1 eEF-2 **kinase** Efp Eg5 EGF Recept EGF Recept (activated) EIF-4 gamma eIF-4E eIF-5A Endoglin Endothelin 1 Recept. EphA4/ELF-1 EPS-8 ERG2 ERp72 v.2 ESE1 v.3 Exportin-1/CRM1 EXPORTIN-t v.3 Ezrin FADD v.2 Fas Fas Ligand FBP v.2 FEN-1 FIN13 FKBP 12 FKBP51 FKBP65 FLAP v.2 Flotilin-2/ESA FNK v.2 Frabin FXR2 v.2 FYB v.2 G3BP GABAbR2 v.2 GAGE GAP 1 m Gelsolin Gephyrin GLUCOCORTICOID GM-CSF GOK GPI-phoslipase D. GRIP GS-15 v.2 GSK 3D GSPT2 GST pI Guanylate **Kinase** HAP1 v.2 HAX-1 hcKROX HDAC3 HDJ-2 HEME OXYGENASE HHR23B HIC-5 v.2 1 HIF-1a HIF1b/ARNT1 hILP (H59520) hILP (H62120) hPRP16 v.2 hPRP17 HRAD9 HS-1 Hsp-90 Hsp10 Hsp110 HSP70 v.2 IFN-.Yen. **Human** IFN-.Yen. Rat **IKKb** IKKg/NEMO IL-10 IL-12p40 IL-12 p70 IL-13 IL-1.beta. IL-2 IL-2 sRd IL-3 IL-4 IL-5 IL-6 IL-8 INHIBITOR 2 iNOS/MAC NOS Integrin .ae butted.5 IP3 rec IQGAP IRS-1 v.6 ISGF3 p48 ITCH JNKK1/MKK4 K CHANNEL a SUB. Kalinin B1 Karyopherin B KATANIN p80 Ki-67 KIF3B v.2 KRIP-1 KSR-1 v.3 Ku70 L-Caldesmon LA PROTEIN LAIR-1 v.2 Lamp1/CD107a LAR-PTP LAT v.2 LCK LFA-1 alpha LSP1 LXRa v.3 M33 MAP4 MCC MCM5 MCP-1 MDC9 Mek5 Melusin MENA MINT1 v.2 MINT3/XII gamma MITOSIN MKK7 v.2 MONA MRE II MSH3 MSH6/GTBP MST-1 MST-3 MUPP1 v.2 MXI-1 MYR6 NABC1/AIBC1 NASP v.2 NEDD-4 Nek 2 Nek3 NES-1 NESTIN Neurexin I NEUROGENIN 3 NEUROGLYCAN v.2 NEUROPILIN-2 NEXILIN NF kappa beta v.2 NHE-1 NHE-3 Nhe-3 v.3 NIP1 NM23 NMDAR2B v.2 nNOS/NOS Type1 NTF2 NUCLEPORIN P62 v.2 NuMA Nup88 p116 RIP v.2 p19 SKP1 p190-B v.3 p36 p38 delta v.2 p47 PHOX p52/LEDGF P53 p54nrb p56 dok2 P62DOK PARP Paxillin PCMT-II PCNA PDGF Rec PDI PECI PER2 Pericentrin Pex1 v.2 PEX19 PhLP PI3-**KINASE** p110a PI4-**Kinase** Beta PIN v.6 PIP5Kg PKC EPSILON PKC iota PKC LAMBDA* PKC THETA PKR v.3 PLAKOPHILIN 2a PLC beta 1 v.2 PLC delta 1 PLK PMF-1 PNUTS PRK2 v.2 R cadherin v.2 RAB 27 RAB-5 RAB11 RAB4 Rabphilin 3A RACK 1 RAP2 RAS (Ha) RAS-GRF2 v.2 RB2 RCH-1 REF-1 RNAse H1 ROAZ v.2 ROK alpha sCD23 SCP3 SH2-B v.2 Shc C SII SIII p15 SIP1 SKAPP55 v.2 SLP76 SMAD2 v.2 SMAD4 SMN v.2 SNX1 v.3 SNX2 SPA-1 v.2 SPOT 14 SQS SRP54 SRPK1 SSeckS STAT-3 STAT6/IL-4 STAT v.5 STI1 SYNAPTOGYRIN Synaptophysin Synaptotagmin Syntaxin 4 SYNTAXIN 6 v.2 TAF 70-alpha TAF-172 Tat-SF1 TENSIN TFII-1 TGF-.beta. THROMBOSPONDIN 1 TIAR TIEG2 TLS TNF-.oe butted. TOPO IIa v.2 TOPO IIb TPL-2 TRADD v.2 TRAX TREX 1 TRF2 TRP1 UBE3A V-1/myotrophin VASP Veli1 VESL-1L VHR VLA-3 alpha VTI1b SUBUNIT WRN XIN XPD ZAP-70K ZBP-89 ZFP-37 ZO-1

PGPUB-DOCUMENT-NUMBER: 20030032055

PGPUB-FILING-TYPE: new

DOCUMENT-IDENTIFIER: US 20030032055 A1

TITLE: Diagnosis and treatment of medical conditions associated with defective NFkappa B(NF-kappaB) activation

PUBLICATION-DATE: February 13, 2003

INVENTOR-INFORMATION:

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Heiss, Nina	Heidelberg		DE	
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APPL-NO: 09/ 863049

DATE FILED: May 22, 2001

RELATED-US-APPL-DATA:

non-provisional-of-provisional 60206223 20000522 US

US-CL-CURRENT: 435/7.1

ABSTRACT:

Incontinentia Pigmenti (IP) is a neurocutaneous genodermatosis that segregates as an X-linked dominant disorder with a high probability of prenatal male lethality. A locus in Xq28 containing NF-.kappa.B Essential Modulator, a gene product involved in the activation of NF-kB and central to many pro-inflammatory and apoptotic pathways, contains mutations in the majority of cases of IP. Disclosed are methods, compositions and kits directed to a defect in a NF-.kappa.B related disease such as IP.

[0001] This application claims priority to U.S. Provisional Patent Application Serial No. 60/206,223, filed May 22, 2000.

---------- KWIC ---------

Summary of Invention Paragraph - BSTX:

[0006] Linkage of the IP locus to markers in distal Xq28 (DXS52-tel) was established with close linkage to the gene for factor VIII (Sefiani, 1989; Smahi, 1994; Parrish, 1996; Jouet, 1997) Many genes from this region have been excluded by extensive mutation screening (Heiss, 1999; Aradhya, 2000; Woffendin, 2000; Das, 1994). Recently, a gene intimately involved in inflammatory responses, NEMO/IKK.gamma., has been mapped 200 kb proximal to the factor VIII locus (Jin, 1999). NEMO is central to the activation of the ubiquitous transcription factor NF-.kappa.B (Yamaoka, 1998; Rudolph, 2000). The NF-.kappa.B/Rel family of transcription factors plays a particularly important role in inflammatory and immune responses, in cellular stress, and in regulating apoptosis (Ghosh, 1998; Baldwin, 1996). Their activity is induced by a variety of different stimuli including pro-inflammatory cytokines such as interleukin-1 (IL-1) and Tumour Necrosis Factor (TNF). The immediate responsiveness required of such a key regulator is effected by an unique mechanism whereby NF-.kappa.B homo- or heterodimers are sequestered in the cytoplasm through interaction with an inhibitory molecule of the IKB family (three different species exist in cells: I.kappa.B.alpha., I.kappa.B.beta. and I.kappa.B.epsilon.). Upon cytokine stimulation the I.kappa.B molecules are phosphorylated on two Ser residues, then polyubiquitinated and degraded through the ubiquitin-proteasome pathway. NF-.kappa.B is therefore free to translocate to the nucleus and to activate its target genes. This phosphorylation event is carried out by a high molecular weight, multiprotein **kinase** complex containing two subunits with **kinase** activity (IKK1/.alpha. and IKK2/.beta.) (Zandi, 1999; Mercurio, 1999). A third component of this complex is a 48 kDa protein with no apparent catalytic activity, called NEMO (NF-.kappa.B Essential Modulator), IKK.gamma., or **IKKAP** (Yamaoka, 1998; Rothwarf, 1998; Mercurio, 1999). NEMO directly interacts with the **kinase** subunits and is required for activation of the **kinase** complex in response to extracellular (or intracellular) stimuli: its absence results in a complete inhibition of NF-.kappa.B activation.

Detail Description Paragraph - DETX:

[0243] NEMO has been shown to play an essential role in the NF-.kappa.B activation process (Yamaoka, 1998). NF-.kappa.B homo- or heterodimers are sequestered in the cytoplasm through interaction with an inhibitory molecule of the IkB family. Upon cytokine stimulation, the IkB molecules are phosphorylated, polyubiquitinated and degraded through the ubiquitin-proteasome pathway (Ghosh et al., 1998; Rothwarf and Karin, 1999). NF-.kappa.B is then free to translocate to the nucleus and to activate its target genes. This phosphorylation event is carried out by a high molecular weight, multiprotein, **kinase** complex containing two subunits with **kinase** activity (IKK1/a and IKK2/b). The third known component of this **IKK** complex is NEMO (**Ikkg, IKKAP or Human** Gene Nomenclature name: IKBKG) a 48 kDa protein with no apparent catalytic activity that directly interacts with the **kinase** subunits and is for activation of the **kinase** complex in response to extracellular (or intracellular) stimuli: its absence results in a complete inhibition of

NF-.kappa.B activation.

Detail Description Paragraph - DETX:

[0252] That NEMO activity is indeed ablated by the rearrangement has been shown for two cell lines expressing this mutation. Can the pathology observed in IP male and female patients therefore be explained in terms of NEMO function? NEMO is a structural/regulatory component of the **IKK** complex that also contains IKK.alpha./1 and IKK.beta./2 (Yamaoka, 1998; Courtois, 1997; Rothwarf, 1998; Mercurio, 1999). Ablation of NEMO activity results in the inability of a cell to activate NF-.kappa.B in response to a series of stimuli (Courtois, 1997; Yamaoka, 1998). The NF-.kappa.B signalling pathway itself has been implicated in immune, inflammatory, and apoptotic responses (for a review see Ghosh, 1998; Baldwin, 1996). Recently, the genes encoding the components of the **IKK** complex, including NEMO, have been inactivated by homologous recombination. Inactivation of **IKK2** (which does not entirely abolish NF-.kappa.B activation, probably due to partial compensation by the **IKK1 kinase** subunit) resulted in embryonic death due to massive liver apoptosis at day 14 (Li, 1999; Li, 1999; Tanaka, 1999) while that of NEMO (which apparently results in a complete block in NF-.kappa.B activation) resulted in death of the male embryos at day 12 with a similar phenotype (**murine** NEMO is also located on the X-chromosome). Apparently the females were normal. A similarly dramatic phenotype was observed when the gene encoding re1A, the most ubiquitously expressed and most potent transcriptional activator of the NF-.kappa.B family, was inactivated: the mice died at embryonic day 15-16 from massive liver apoptosis (Beg, 1995). Interestingly this apoptosis is due to the pro-apoptotic effect of TNF, as demonstrated by the viability of mice carrying an inactivation of both the re1A and the TNF genes (Doi, 1999). This observation is in keeping with the high sensitivity to TNF-induced apoptosis in cell lines derived from IP patients (FIG. 5D). In both mice and humans, therefore, the complete absence (or lack of activity) of NEMO results in early lethality of the affected males. Post mortem examination of a few **human** affected males has suggested the involvement of an abnormal immune response, as expected from a defect in NF-.kappa.B signalling.

Detail Description Paragraph - DETX:

[0255] Another interesting hint comes from the analysis of mice carrying an inactivation of the **IKK1** component of the complex that includes NEMO (Hu, 1999; Li, 1999; Takeda, 1999). Inactivation of this **kinase** results in a completely unexpected phenotype: the resulting mice exhibit an almost intact activation of NF-.kappa.B by pro-inflammatory stimuli but show multiple defects in morphogenetic events, including limb and skeletal patterning and proliferation and differentiation of epidermal keratinocytes. Such an epidermal phenotype has also been observed with transgenic mice expressing a dominant negative version of I.kappa.Ba under skin-specific promoter control (Seitz, 2000; van Hogerlinden, 1999; Seitz, 1998). At the skin level NF-.kappa.B appears to play a dual role: it controls cell growth in the stratified epithelium and regulates apoptosis. Defect in both pathways may explain the characteristic skin lesions observed in IP2.

Detail Description Paragraph - DETX:

[0345] Hu, Y. L. et al. Abnormal morphogenesis but intact **IKK** activation in mice lacking the IKK.alpha. subunit of I.kappa.B **kinase**. Science 284, 316-320 (1999).

Detail Description Paragraph - DETX:

[0359] Mercurio, F. et al. I.kappa.B **kinase (IKK)**-associated protein 1, a common component of the heterogeneous **IKK** complex. Mol Cell Biol 19, 1526-1538 (1999).

Detail Description Paragraph - DETX:

[0366] Rothwarf, D. M., Zandi, E., Natoli, G. & Karin, M. **IKK**-.gamma. is an essential regulatory subunit of the I.kappa.B **kinase** complex. Nature 395, 297-300 (1998).

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ABSTRACT:

The present invention describes methods of preventing and treating inflammatory and immune-related diseases or disorders using inhibitors of I.kappa.B **kinase (IKK)**. Also described are **IKK** inhibitors effective for the prevention and treatment of inflammatory and immune-related diseases or disorders, as demonstrated in vivo. Further embodiments of the present invention relate to a specific **IKK** inhibitors, 4(2'-aminoethyl)amino-1,8-- dimethylimidazo(1,2-a) quinoxaline and compounds of formula (I), salts thereof, and pharmaceutical compositions.

RELATED INVENTIONS

[0001] This application claims benefit to application U.S. Serial No. 60/223,304, filed Oct. 3, 2000 and application U.S. Serial No. 60/265,853, filed Feb. 1, 2001, and is a continuation-in-part of U.S. Ser. No. 09/965,977, filed Sep. 27, 2001. The contents of all of which are hereby incorporated by reference herein in their entireties.

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Abstract Paragraph - ABTX:

The present invention describes methods of preventing and treating inflammatory and immune-related diseases or disorders using inhibitors of I.kappa.B **kinase (IKK)**. Also described are **IKK** inhibitors effective for the prevention and treatment of inflammatory and immune-related diseases or disorders, as demonstrated in vivo. Further embodiments of the present invention relate to a specific **IKK** inhibitors, 4(2'-aminoethyl)amino-1,8-- dimethylimidazo(1,2-a) quinoxaline and compounds of formula (I), salts thereof, and pharmaceutical compositions.

Title - TTL:

Methods of treating inflammatory and immune diseases using inhibitors of IkappaB **kinase (IKK)**

Summary of Invention Paragraph - BSTX:

[0002] The present invention relates to methods of using inhibitors of I.kappa.B **kinase (IKK)** in the treatment of inflammatory and immune diseases, to inhibitors of **IKK,** and to pharmaceutical compositions comprising such inhibitors, together with a pharmaceutically or physiologically-acceptable vehicle or excipient.

Summary of Invention Paragraph - BSTX:

[0007] Potential inhibitors of NF-.kappa.B and/or the NF-.kappa.B pathway have been identified as including Interleukin-10, glucocorticoids, salicylates, nitric oxide, and other immunosuppressants. I.kappa.B is a cytoplasmic protein that controls NF-.kappa.B activity by retaining NF-.kappa.B in the cytoplasm. I.kappa.B is phosphorylated by the I.kappa.B **kinase (IKK)**, which has two isoforms, **IKK-1** (or I.kappa.B **kinase** .alpha., IKK.alpha.) and **IKK-2** (or I.kappa.B **kinase** .beta., IKK.beta.). Upon phosphorylation of I.kappa.B by **IKK,** NF-.kappa.B is rapidly released into the cell and translocates to the nucleus where it binds to the promoters of many genes and up-regulates the transcription of pro-inflammatory genes. Inhibitors of **IKK** can block the phosphorylation of I.kappa.B and further downstream effects, specifically those associated with NF-.kappa.B transcription factors. Glucocorticoids reportedly inhibit NF-.kappa.B activity by two mechanisms, i.e., upregulating I.kappa.B protein levels and inhibiting NF-.kappa.B subunits. Nitric oxide also reportedly inhibits NF-.kappa.B through upregulation of I.kappa.B. However, these mechanisms of interaction are complex; for example, production of nitric oxide in lymphocytes reportedly enhances NF-.kappa.B activity.

Summary of Invention Paragraph - BSTX:

[0010] I.kappa.B **kinase (IKK)** is a high molecular weight (500-900 kD) multisubunit enzyme which phosphorylates I.kappa.B-.alpha. at positions serine-32 and serine-36 and has been isolated from HeLa cells (Chen et al. Cell 84:853-862,1996; Lee et al. Cell 88:213-222,1997; DiDonato et al. Nature 388:548-554, 1997). Two catalytic subunits termed **IKK-1 and IKK-2 of IKK** have been identified, cloned, and demonstrated to be widely expressed in **human** tissues (DiDonato et al. Nature 388:548-554,1997; Zandi et al. Cell 91:243-252,1997; Mercurio et al. Science 278:860-866,1997; Woronicz et al. Science 278:866-869, 1997; Li et al. J. Biol. Chem. 273:30736-30741, 1998; Regnier et al. Cell 90:373-383,1997).

Summary of Invention Paragraph - BSTX:

[0011] The **IKK-1 and IKK-2** catalytic subunits of **IKK** are highly homologous, having 50% sequence identity and more than 70% sequence similarity. **IKK-1 and IKK-2** are 85- and 87-kDa proteins, respectively. Both **kinases** contain a catalytic domain followed by a leucine zipper domain and a helix-loop-helix (HLH) domain (Mercurio et al. Science, 278:860-866, 1997). When one subunit is recombinantly expressed without the other subunit, either one is still able to catalyze the phosphorylation of I.kappa.B (Li et al. J. Biol. Chem., 273:30736-30741, 1998). Thus, **IKK,** either **IKK-1 or IKK-2,** can play an important role in signaling I.kappa.B for ubiquination and further degradation.

Summary of Invention Paragraph - BSTX:

[0012] In addition, in vitro studies have demonstrated that the full length IKK.beta. can autophosphorylate and phosphorylate its substrate, I.kappa.B.alpha., as well; however, neither the N-terminal **kinase** domain-containing form, nor the C-terminal HLH domain-containing form of **IKK** was capable of autophosphorylation (U.S. Pat. No. 6,077,701 to Chu et al.). U.S. Pat. No. 6,077,701 discloses that compounds which increase IKK.beta. activity and/or binding to I.kappa.B can be potential modulators of inflammatory disease.

Summary of Invention Paragraph - BSTX:

[0014] There are other **kinases** which can phosphorylate I.kappa.B and which have been implicated in the activation of NF-.kappa.B. For example, two **kinases** (pp90rsk and **IKK**-.epsilon.) have been demonstrated to phosphorylate I.kappa.B-.alpha. at serine-32 and/or serine-36. The overexpression of these **kinases,** and the use of dominant negative mutants to these **kinases,** have indicated a role for them in the phosphorylation of I.kappa.B in cells (Ghoda et al., J. Biol. Chem. 272:21281-21288, 1997; Peters et al., Mol. Cell. 5:513-522, 2000). The existence of multiple I.kappa.B **kinases** is indicative of redundant signaling pathways. Therefore, it is possible that an inhibitor of IKK-1 and/or IKK-2 can not necessarily show anti-inflammatory or immunosuppressive effects due to redundant signaling pathways in at least some

cells.

Summary of Invention Paragraph - BSTX:

[0015] Several in vitro types of studies have been performed to further investigate **IKK** and its properties. The in vitro types of cell biology studies (for example, overexpression of either **IKK-1 or IKK-2,** or of dominant negative versions of these **kinases** (DiDonato et al. Nature 388:548-554,1997; Mercurio et al. Science 278:860-866,1997; Woronicz et al. Science 278:866-869,1997; Regnier et al. Cell 90:373-383; Zandi et al. Cell 91:243-252)) that appear to implicate **IKK-1 and IKK-2** in NF-.kappa.B activation are sometimes artifactual. A particular example of such an artifactual study involves a **kinase** known as NF-.kappa.B-inducing **kinase,** NIK.

Summary of Invention Paragraph - BSTX:

[0016] Overexpression of NIK in cells activated **IKK** and NF-.kappa.B, while expression of **kinase**-inactive forms of the enzyme blocked the stimulated activation of **IKK** and NF-.kappa.B (Malinin et al., Nature 385:540-544, 1997; Song et al., Proc. Nat. Acad. Sci. USA 94:9792-9796, 1997). Based on the foregoing, as well as on yeast two-hybrid studies showing a strong interaction between NIK and **IKK** (Regnier et al., Cell 90:373-383, 1997), NIK is suggested to be essential for the activation of **IKK** and, subsequently, NF-.kappa.B.

Summary of Invention Paragraph - BSTX:

[0017] Additional studies have demonstrated that the NIK-**IKK** protein-protein interaction is important for NF-.kappa.B-dependent responses (WO99/43704; Publication Date: Sept. 2, 1999; Goeddel et al., U.S. Pat. Nos.: 5,851,812; 5,916,760; and 5,939,302). Goeddel et al. show that transient IKK.beta. overexpression induces luciferase reporter gene activity in both HeLa and 293 cells and overexpression of **kinase**-inactive IKK.beta., which still associates with NIK, blocks activation. However, it was further determined that cells derived from mice deficient in NIK had no abrogated NF-.kappa.B activation (Karin and Ben-Nariah, Ann. Rev. Immunol., 18:621-663, 2000). Therefore, although some studies suggest the importance of NIK as a potential target for inhibiting NF-.kappa.B activation, the experimental results were not demonstrable in vivo, as reported by Karin and Ben-Nariah (supra). Thus, it is very important that in vitro-based and strictly cell biology-based experiments using overexpressed proteins always be cautiously interpreted.

Summary of Invention Paragraph - BSTX:

[0026] In animal models of disease (i.e. knock-out mice) designed to test inhibition of **IKK,** the deletion of **IKK-1 or IKK-2** has been demonstrated to be embryonic lethal (Li et al. Science 284:321-325, 1999; Hu et al. Science 284:316-320, 1999). Therefore, the use of IKK knockout mice to demonstrate a role of **IKK** in disease is neither practical nor feasible. In contrast, the

present invention demonstrates for the first time that inhibitors of the catalytic activity of **IKK-1 and IKK-2** are effective in **murine** models of disease. Such models are believed to be predictive of similar effects in **human** patients. Therefore, the treatment methods, in vivo models, and effectiveness of **IKK** inhibitors as described herein, are extremely beneficial and advantageous for the advancement of discovering and employing therapeutics for inflammation and immune system diseases. These animal models are therefore important tools for studying **human** diseases, specifically inflammatory and immune-related diseases.

Summary of Invention Paragraph - BSTX:

[0030] The present invention relates to methods of preventing and treating inflammatory and immune-related diseases or disorders using I.kappa.B **kinase (IKK)** inhibitors, compounds of formula (I) as described herein. Also provided is the **IKK** inhibitor 4(2'-aminoethyl)amino-1,8-dimethylimid- azo(1,2-a) quinoxaline used to prevent and treat inflammatory and immune-related diseases or disorders.

Detail Description Paragraph - DETX:

[0054] The present invention describes methods of preventing and treating inflammatory and immune-related diseases or disorders using inhibitors of I.kappa.B **kinase (IKK)**. Such methods are efficacious in vivo in inhibiting **IKK** activity in mammalian, including **human** and biological systems, e.g. animal models of disease.

Detail Description Paragraph - DETX:

[0062] The term "**IKK**" as used herein, refers to I.kappa.B**-kinase (IKK)**. **IKK** comprises two catalytic subunits, **IKK-1 and IKK-2,** also known as IKK.alpha. and IKK.beta., respectively.

Detail Description Paragraph - DETX:

[0063] "Activation of **IKK**" as used herein means changing an inactive **IKK** protein into an active **IKK** protein that functions as an I.kappa.B **kinase**. Activated **IKK** phosphorylates serine-32 and serine-36 of I.kappa.B, which thus marks the I.kappa.B for ubiquitination and degradation.

Detail Description Paragraph - DETX:

[0100] According to the present invention, compounds which inhibit **IKK,** i.e. **IKK** inhibitors, can be used in the methods for preventing and treating inflammatory and immune system-related conditions, diseases, or disorders. Non-limiting examples of such diseases, disorders, and conditions include arthritis, rheumatoid arthritis, psoriatic arthritis, osteoarthritis, acute

pancreatitis, chronic pancreatitis, psoriasis, glomerulonephritis, serum sickness, lupus (systematic lupus erythematosis), urticaria, scieraclerma, contact dermatitis, dermatomyositis, alopecia, atopic eczemas, ichthyosisrhinitis, inflammatory bowel disease (Crohn's and ulcerative colitis); Alzheimer's disease, brain ischemia, traumatic brain injury, Parkinson's Disease, Creutzfeldt-Jacob diseases, HIV encephalitis, cerebral malaria, and meningitis, atherosclerosis, ataxia telangiectasis, septic shock, multiple sclerosis, amyotrophic lateral sclerosis, atherosclerosis, subarachnoid hemorrhage, autoimmune diseases, systemic lupus erythematosus, multiple sclerosis, hematopoiesis, pulmonary diseases, respiratory allergies, asthma, acute respiratory distress syndrome, hayfever, allergic rhinitis, allergic respiratory disease, herpes simplex type 1 (HSV-1), herpes simplex type 2 (HSV-2), cytomegalovirus, Epstein-Barr, **human** immunodeficiency virus (HIV), Addison's disease (autoimmune disease of the adrenal glands), idiopathic adrenal insufficiency, autoimmune polyglandular disease (also known as autoimmune polyglandular syndrome), fungal infections, mycosis fungoides, chronic obstructive pulmonary disorder; tissue/organ transplant rejection (e.g., kidney, liver, heart, lung, pancreas, bone marrow, cornea, small bowel, skin allografts, skin homografts, and heterografts, etc.); stroke, oncological diseases, cancer, tumors, breast cancer, prostate cancer, and Hodgkin's lymphoma.

Detail Description Paragraph - DETX:

[0108] One embodiment of the present invention provides beneficial and advantageous in vivo animal studies using **IKK** inhibitors that demonstrate compound efficacy using methods of the present invention. For example, 4 (2'-aminoethyl)amino-1,8-dimethylimidazo(1,2-a) quinoxaline, compound 6 herein, or a salt thereof, newly discovered by the present inventors, inhibits the catalytic activity of I.kappa.B **kinase** by blocking the active site of **IKK** which is responsible for phosphorylating the I.kappa.B protein. Moreover, the examples described herein demonstrate that **IKK** inhibitors are particularly useful in methods of treating inflammatory and immune-related diseases or disorders in several different animal models of disease that are considered to be predictive of similar employment and effects in **human** patients.

Detail Description Paragraph - DETX:

[0169] A therapeutically effective dose refers to that amount of active ingredient or compound, such as an **IKK** inhibitor, which ameliorates, reduces, or eliminates the symptoms, condition, disease, or disorder. Therapeutic efficacy and toxicity can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., ED50 (the dose therapeutically effective in 50% of the population) and LD50 (the dose lethal to 50% of the population). The dose ratio of therapeutic to toxic effects is the therapeutic index, which can be expressed as the ratio, ED50/LD50. Pharmaceutical compositions which exhibit large therapeutic indices are preferred. As understood by the skilled practitioner, data obtained from cell culture assays and animal studies are used in determining a range of dosages for **human** use. Preferred dosage of a pharmaceutical composition is within a range of circulating concentrations that include the ED.sub.50 with little or no

toxicity. The dosage varies within this range depending upon the dosage form employed, sensitivity of the patient, and the route of administration.

Detail Description Paragraph - DETX:

Effect of **IKK** Inhibitor 4(2'-Aminoethyl)amino-1,8-dimethylimidazo(1,2-a)quinoxaline (Compound 6) on Disease Incidence in the **Murine** Model of Collagen-Induced Arthritis

Detail Description Paragraph - DETX:

[0218] The **murine** model of collagen-induced arthritis shares many of the same biochemical and pathological mechanisms as does rheumatoid arthritis in humans, and was used for preventing and treating arthritis upon the administration of 4(2'-aminoethyl)amino-1,8-dimethylimidazo(1,2-a) quinoxaline (compound 6). FIGS. 1 and 2 and Table 5 show that daily administration of 30 mg/kg (p.o.) of compound 6 inhibited disease onset, and that 100 mg/kg (p.o.) of compound 6 prevented any disease onset when dosed in a preventative mode. When the **IKK** inhibitor was administered after disease onset (i.e., established mode of therapy), FIG. 3 and Table 6 show that treatment with 30 mg/kg (p.o.) of compound 6 significantly inhibited disease progression when compared with vehicle treated animals, while a dose of 100 mg/kg (p.o.) of compound 6 actually led to significant disease resolution. These results support the advantages provided by the present invention in which inflammatory diseases or disorders, such as rheumatoid arthritis, are prevented or ameliorated by inhibiting **IKK** activity.

Detail Description Paragraph - DETX:

Effect of **IKK** Inhibitor 4(2'-Aminoethyl)amino-1,8-dimethylimidazo(1,2-a)Quinoxaline on Disease Incidence in the **Murine** Model of Dextran Sulfate Sodium-Induced Inflammatory Bowel Disease

Detail Description Paragraph - DETX:

[0228] The results show that 4(2'-aminoethyl)amino-1,8-dimethylimidazo(1,2- -a) quinoxaline (compound 6) prevented and treated IBD in the well-established **murine** model of inflammatory bowel disease (IBD) in humans. Tables 11 and 12 show that daily administration of the **IKK** inhibitor (compound 6) resulted in a statistically significant decrease in clinical and histological score when compared with untreated mice. Such results support the advantageous outcome afforded by the present invention in which inhibition of the **IKK** enzyme prevents and/or ameliorates inflammatory diseases and disorders such as IBD.

Detail Description Paragraph - DETX:

Effect of **IKK** Inhibitor 4(2'-Aminoethyl)amino-1,8-dimethylimidazo(1,2-a)qu-

inoxaline on Disease Incidence in the **Murine** Model of Ovalbumin-Induced Inflammatory Cell Infiltration in the Lung of Sensitized Mice

Detail Description Paragraph - DETX:

[0230] Pulmonary inflammation with infiltration of eosinophils and other inflammatory cells is a hallmark of asthma and other allergic respiratory disorders. To determine whether an **IKK** inhibitor was efficacious in the in vivo treatment of respiratory disorders which afflict many patients, test compounds were administered in a **murine** model of pulmonary inflammation similar to that described by Kung et al. (Kung, T. T. et al., Int. Arch. Allergy Immunol. 105:83-90,1994).

Detail Description Paragraph - DETX:

[0232] Ovalbumin-induced inflammatory cell infiltration in **murine** lung provides a model system for pulmonary inflammation, specifically asthma and other allergic respiratory disorders in humans. Compound 6, 4(2'-aminoethyl)amino-1,8-dimethylimidazo(1,2-a) quinoxaline, was administered in a **murine** model of pulmonary inflammation for the treatment of inflammation and immune-related conditions. The results presented in Table 13 show that administration of compound 6 after ovalbumin challenge to sensitized mice resulted in a statistically significant decrease in total inflammatory cells in the lungs, as determined by measurements from bronchoalveolar lavage fluid when compared with untreated mice. These results support the advantages provided by the present invention in which inflammatory diseases or disorders, such as asthma and chronic obstructive pulmonary disease, are ameliorated by inhibiting **IKK** activity.

Claims Text - CLTX:

29. A method of treating an inflammatory or immune-related disease or disorder comprising administering to a mammal in need thereof, an **IKK** inhibitor in an amount effective to inhibit I.kappa.B **kinase (IKK), wherein IKK** inhibition results in one or more of the following: i) inhibition of **IKK** catalytic activity; ii) inhibition of phosphorylation of I.kappa.B; or iii) inhibition of NF-.kappa.B-dependent gene expression.

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ABSTRACT:

A method of enhancing bone formation comprising administering an effective amount of 1) an oligomeric complex of one or more of RANKL, a RANKL fusion protein or an analog, derivative or mimic thereof, 2) an osteogenic compound capable of enhancing activity of one or more intracellular proteins in osteoblasts or osteoblast precursors, wherein said activity is indicative of bone formation, or 3) an osteogenic compound capable of inactivating one or more phosphatases in osteoblasts or osteoblast precursors, wherein said inactivation is indicative of bone formation. The method also may be used to treat a disease or condition manifested at least in part by the loss of bone mass by administering to a patient a pharmaceutical composition comprising an oligomeric complex or osteogenic compound disclosed herein.

[0001] This application claims the benefit of U.S. Provisional Applications Ser. Nos. 60/277,855, 60/311,163, 60/329,231, 60/328,876, and 60/329,393, filed Mar. 22, 2001, Aug. 9, 2001, Oct. 12, 2001, Oct. 12, 2001, and Oct. 15, 2001, respectively, all of which are hereby incorporated herein by reference.

---------- KWIC ---------

Summary of Invention Paragraph - BSTX:

[0016] In one embodiment, intracellular proteins are selected from IKB-.alpha. and IKB-.beta.. In a preferred embodiment, the intracellular proteins exhibiting prolonged activity comprise intracellular **kinases,** and more preferably such **kinases** are ERK1/2, **IKK,** PI3 **kinase,** Akt, JNK, and p38. In a more preferred embodiment, the **kinases** are ERK1/2.

Summary of Invention Paragraph - BSTX:

[0017] In another preferred embodiment, the activity of one or more intracellular proteins constitutes phosphorylation of said protein(s). Specifically, the phosphorylated proteins include ERK1/2, **IKK,** P13 **kinase,** Akt, JNK, and p38. More preferably, the phosphorylated **kinases** are ERK1/2.

Summary of Invention Paragraph - BSTX:

[0019] In another embodiment, osteogenic compounds capable of inactivating one or more phosphatases in osteoblasts or osteoblast precursors, wherein said inactivation is indicative of bone formation may be used in the methods and compositions of the present invention. Preferably, said phosphatase is selected from the group consisting of ERK1-, ERK2**-, IKK**-, P13 **kinase**-, Akt-, JNK-, and p38-specific phosphatases, and more preferably the phosphatese is specific for ERK1/2. In another preferred embodiment, inactivation comprises phosphorylation of a phosphatase.

Detail Description Paragraph - DETX:

[0052] "**IKK**" is an abbreviation for IkappaB (IKB) **kinase**.

Detail Description Paragraph - DETX:

[0091] In a further embodiment, applicants have discovered that the interaction between oligomeric RANKL and its receptor RANK on osteoblasts or osteoblast precursors results in prolonged intracellular activity of intracellular proteins. **Mouse** osteoblasts, when treated with GST-RANKL in vitro manifested activation, as characterized by the activation of NF.kappa.B and ERK intracellular signal pathways. As noted by the applicants, the time course of intracellular protein activity, especially ERK activity is different from that observed in osteoclast precursors, which also express RANK on the surface. In osteoclast precursors, ERK activity peaks 5-15 minutes after RANK/GST-RANKL interaction, and returns to basal levels after 15-30 minutes. In contrast, the ERK activity in osteoblasts peaks at 10 minutes after the same interaction, and is still above the basal level after 60 minutes. The prolongation of the time course is even more prominent in osteoblast precursor cells, wherein the

demonstrated activity of ERK had not reached its maximum even 60 minutes after the RANK/oligomeric GST-RANKL interaction. Besides the different time course of ERK activity, osteoblasts and osteoblast precursor cells also exhibit prolonged activity of **kinases such as IKK,** P13 **kinase,** Akt, p38 and JNK. This osteoblast-related activity contrasts with GST-RANKL interaction with RANK on osteoclasts, which results in short-lived activity of MAP **kinases** and bone resorption. While not being bound to a particular theory, it therefore appears that the prolonged activity of **kinases** observed in osteoblasts following oligomeric GST-RANKL stimulation plays a role in the anabolic bone processes.

Detail Description Paragraph - DETX:

[0093] Accordingly, osteogenic compounds capable of enhancing activity of one or more intracellular proteins in osteoblasts or osteoblast precursors, wherein such activity is indicative of bone formation, may be used in the methods of the present invention Activated intracellular proteins include but are not limited to **kinases**. Preferably, the **kinases** comprise ERK1/2, JNK, P13 **kinase, IKK,** Akt, and p38, and even more preferably, the **kinases** are ERK1/2. Other intracellular proteins include IKB-.alpha. and IKB-.beta..

Detail Description Paragraph - DETX:

[0102] In another embodiment, osteogenic compounds capable of inactivating one or more phosphatases in osteoblasts or osteoblast precursors, wherein said inactivation is indicative of bone formation may be used in the methods of the present invention. In one preferred embodiment, the phosphatases inhibit the **kinases** involved in osteogenesis, including p38, ERKs, JNK, **IKK,** and Akt. More preferably, the phosphatases are MAPK specific or Akt specific, and even more preferably they are ERK1/2 specific. While not being bound to a particular theory, this method is feasible for this purpose due to the fact that a **kinase** activity is tightly regulated by its corresponding phosphatase. In case of ERK1/2, the phosphatase is known as the mitogen activated protein **kinase** phosphatase-3 (MKP-3). This phosphatase belongs to a family of dual specificity phosphatases, which are responsible for the removal of phosphate groups from the threonine and tyrosine residues on their corresponding **kinases** (Camps et al., FASEB J., 14, pp.6-16, 1999). The prompt removal of phosphate groups by phosphatases ensures that **kinase** activation is short-lived and that the level of phosphorylation is low in a resting cell. However, in order for the phosphatase to be active and remove phosphate groups, it also needs to be phosphorylated. Therefore, inhibition of phosphatase activity results in activation or prolongation of ERK1/2 activity.

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PGPUB-FILING-TYPE: new

DOCUMENT-IDENTIFIER: US 20030013125 A1

TITLE: Novel ligands and libraries of ligands

PUBLICATION-DATE: January 16, 2003

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DATE FILED: May 10, 2002

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child 10143455 A1 20020510 parent continuation-in-part-of 10121216 20020410 US PENDING child 10121216 20020410 US parent continuation-in-part-of 09981547 20011017 US PENDING child 09981547 20011017 US parent division-of 09105372 19980626 US GRANTED parent-patent 6335155 US child 09981547 20011017 US parent division-of 09990421 20011121 US PENDING non-provisional-of-provisional 60252294 20001121 US

US-CL-CURRENT: 435/7.1,436/518 ,558/232 ,560/157 ,560/24 ,564/162 ,564/32 ,564/85

ABSTRACT:

The present invention provides compounds and libraries of compounds having formula (I): 1

wherein L, n, S and A are defined generally and subsets herein. These compounds and libraries of compounds are useful generally in the drug discovery process.

PRIORITY INFORMATION

[0001] This application is a continuation-in-part of U.S. Ser. No. 10/121,216 filed Apr. 10, 2002. The '216 application is a continuation-in-part of U.S. Ser. No. 09/981,547 filed Oct. 17, 2001 which is a divisional of U.S. Ser. No. 09/105,372 filed Jun. 26, 1998, and is a continuation-in-part of U.S. Ser. No. 09/990,421 filed Nov. 21, 2001 which asserts priority to U.S. Provisional Application No. 60/252,294 filed Nov. 21, 2000. All of these priority applications are incorporated herein by reference.

---------- KWIC ---------

Detail Description Paragraph - DETX:

[0132] Preferred protein targets include: cell surface and soluble receptor proteins, such as lymphocyte cell surface receptors; enzymes; proteases (e.g., aspartyl, cysteine, metallo, and serine); steroid receptors; nuclear proteins; allosteric enzymes; clotting factors; **kinases** (serine/threonine **kinases** and tyrosine **kinases**); phosphatases (serine/threonine, tyrosine, and dual specificity phosphatases, especially PTP-1B, TC-PTP and LAR); thymidylate synthase; bacterial enzymes, fungal enzymes and viral enzymes (especially those associated with HIV, influenza, rhinovirus and RSV); signal transduction molecules; transcription factors; proteins or enzymes associated with DNA and/or RNA synthesis or degradation; immunoglobulins; hormones; and receptors for various cytokines. Illustrative examples of receptors include for example, erythropoietin (EPO), granulocyte colony stimulating (G-CSF) receptor, granulocyte macrophage colony stimulating (GM-CSF) receptor, thrombopoietin (TPO), interleukins, e.g. IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-11, IL-12, growth hormone, prolactin, **human** placental lactogen (LPL), CNTF, oncostatin, RANTES, MIPb, IL-8, insulin, insulin-like growth factor 1 (IGF-1), epidermal growth factor (EGF), heregulin-a and heregulin-b, vascular endothelial growth factor (VEGF), placental growth factor (PLGF), tissue growth factors (TGF-a and TGF-.beta.), and nerve growth factor (NGF). Other targets include various neurotrophins and their ligands, other hormones and receptors such as, bone morphogenic factors, follicle stimulating hormone (FSH), and luteinizing hormone (LH), CD40 ligand, apoptosis factor-i and -2 (AP-1 and AP-2), p53, bax/bc12, mdm2, caspases (1, 3, 8 and 9), cathepsins, IL-1/IL-1 receptor, BACE, HIV integrase, PDE IV, Hepatitis C helicase, Hepatitis C protease, rhinovirus protease, tryptase, cPLA (cytosolic Phospholipase A2), CDK4, c-jun **kinase,** adaptors such as Grb2, GSK-3, AKT, MEKK-1, PAK-1, raf, TRAF's 1-6, Tie2, ErbB 1 and 2, FGF, PDGF, PARP, CD2, C5a receptor, CD4, CD26, CD3, TGF-alpha, NF-kB, **IKK** beta, STAT 6, Neurokinnin-1, CD45, Cdc25A, SHIP-2, **human** p53, bax/bc12, IgE/IgER, ZAP-70, ick, syk, ITK/BTK, TACE, Cathepsin S, K and F, CD11a, LFA/ICAM, VLA-4, CD28/B7, CTLA4, TNF alpha and beta, (and the p55 and p75 TNF receptors), CD40L, p38 map **kinase,** IL-2, IL-4, 11-13, IL-15, Rac 2, PKC theta, IL-8, TAK-1, jnk, **IKK2** and IL-18.

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PGPUB-FILING-TYPE: new

DOCUMENT-IDENTIFIER: US 20030004192 A1

TITLE: Method of modulating NF-kB activity

PUBLICATION-DATE: January 2, 2003

INVENTOR-INFORMATION:

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APPL-NO: 10/ 153800

DATE FILED: May 24, 2002

RELATED-US-APPL-DATA:

non-provisional-of-provisional 60292927 20010524 US

US-CL-CURRENT: 514/353,514/336

ABSTRACT:

A method of modulating the level of activated, NF-.kappa.B in cells by contacting cells with a cyanoguanidine compound of general formula I 1

wherein

n is 0, 1 or 2;

each R independently represents halogen, trifluoromethyl, hydroxy, C.sub.1-4 alkyl, C.sub.1-4 alkoxy, C.sub.1-4 alkoxycarbonyl, nitro, cyano, amino, sulfo or carboxy groups;

Q is a straight or branched, saturated or unsaturated C.sub.4-20 divalent hydrocarbon radical;

X Is a bond, O, S, amino, carbonyl, carbonylamino, aminocarbonyl, oxycarbonyloxy, oxycarbonyl, carbonyloxy, aminocarbonyloxy, aminothlocarbonyloxy, oxycarbonylamino or oxythiocarbonylamino;

A is di-(C.sub.1-4 alkoxy)phosphinoyloxy, C.sub.1-4 alkoxycarbonyl, C.sub.1-4 alkoxycarbonylamino, saturated or unsaturated C.sub.3-12 carbocyclic ring or C.sub.3-12 heterocarbocyclic ring optionally substituted with one or more R.sub.1; R.sub.1 being independently selected from the group consisting of halogen, trifluoromethyl, hydroxy, C.sub.1-4 alkyl, C.sub.1-4 alkoxy, C.sub.1-4

alkoxycarbonyl, nitro, cyano, amino, carboxy, sulfo, carboxamido, sulfamoyl or C.sub.1-4 hydroxyalkyl;

or a pharmaceutically acceptable salt, N-oxide or N-substituted prodrug thereof, in an amount effective to modulate the activity of IKK.

---------- KWIC ---------

Summary of Invention Paragraph - BSTX:

[0011] At the cellular level it is well recognised that nuclear factor .kappa.B (NF-.kappa.B) plays a pivotal role in apoptosis. It is also described that an NF-.kappa.B inhibitor, I.kappa.B, and an I.kappa.B **kinase** complex, **IKK,** control the level of activated NF.kappa.B [Levkau, 1, 227-233, 1999; Wang, Science, 274, 784-787, 196; Madrid, Molecular and Cellular Biology, 5, 1626-1638, 2000]. Accordingly, the NF-.kappa.B-I.kappa.B-**IKK** system has been suggested as a target in the treatment of neoplastic diseases.

Summary of Invention Paragraph - BSTX:

[0015] It has surprisingly been found that a certain class of cyanoguanidine derivatives is capable of modulating the activity of I.kappa.B **kinase** (abbreviated **IKK** in the following). By modulating the activity of **IKK** in the cells it is possible to control the level of activated NF-.kappa.B in the cells. Such cyanoguanidines are therefore considered useful in the treatment of neoplastic diseases and other conditions believed to be affected by the level of activated NF.kappa.B, e.g. inflammation.

Detail Description Paragraph - DETX:

[0061] NF-.kappa.B is a member of the Rel family of transcription factors which are ubiquitous in animal cells. Rel proteins can form dimers, the most common of which is designated NF-.kappa.B, NF-.kappa.S is a p50/p65 heterodimer which can activate transcription of genes containing the appropriate .kappa.B binding site. In non-stimulated cells, NF-.kappa.B is maintained in the cytoplasm by interaction with NF-.kappa.B inhibiting proteins, the I.kappa.Bs. In response to cell stimulation by e.g. anti-neoplastic drugs or ionising radiation an I.kappa.B **kinase** complex (**IKK**) is rapidly activated and phosporylates two serine residues in the NF-.kappa.B binding domain of I.kappa.B. The phophorylated I.kappa.B is then degraded by a 26S proteasome whereas NF-.kappa.B is spared from degradation and translocates into the nucleus [Wang, Science, 274, 784-787, 1996, Cusak, Cancer Research, 60, 2323-2330, 2000; Karin, Immunology, 12, 2000, 85-98]. NF-.kappa.B is thus always present in the cell, but in an inactivated form in non-stimulated cells. After translocation into the nucleus NF-.kappa.B induces inter alia the anti-apoptotic genes c-IAP1, c-IAP2, TRAF1, TRAF2, Bfl-1/A1, Bcl-X.sub.L and Mn-SOD [Patel, Oncogene, 19, 2000, 4159-41699], which bring about resistance in the cells to apoptosis. This effect is referred to as the anti-apoptotic effect of NF-.kappa.B, and the effect may be quantified by measuring the expression of

gene products encoded by any of said genes, by any suitable means known in the art, in the presence and absence of compounds modulating the level of activated NK-.kappa.B. Any compound capable of reducing the transcription of one or more of said genes to a level of less than about 50%, e.g. less than about 30%, such as less than about 20% of the level in the absence of said compound is said to reduce the anti-apoptotic effect of NK-.kappa.B. Anti-neoplastic drugs and ionising radiation thus induce resistance in the cells to the treatments, which render them ineffective. Accordingly, activated NF-.kappa.B is a key factor in induced resistance in e.g. cancer cells to chemotherapeutic drugs and/or to ionising radiation, This is further supported by the fact that constitutively activated NF-.kappa.B is found in cells from resistant cancer tumours [Patel, Oncogene, 19, 4159-4169, 2000]. Regardless of reduced resistance to any treatment, a reduction of the level of activated NF-.kappa.B In the cell, e.g. by controlling the activity of **IKK,** will reduce the expression levels of genes encoding for anti-apoptotic factors, thereby inducing apoptosis in the cells [Schwartz, Surgical Oncology, 8, 1999, 143-153].

Detail Description Paragraph - DETX:

[0064] The I.kappa.B **kinase** complex (**IKK**) consist of three subunits, namely IKK.alpha., IKK.beta. and IKK.gamma., with a combined molecular weight of 900 kDa. IKK.alpha. and IKK.beta. both exhibit I.kappa.B **kinase** activity and phophorylate I.kappa.B, whereas IKK.gamma. is a regulatory subunit. IKK.alpha. is 85 kDa protein and IKK.beta. is a 87 kDa protein, and the two subunits show a large degree of homology. Whereas both IKK.alpha. and IKK.beta. are catalytically active, it has surprisingly been shown that only IKK.beta. is essential for **IKK** phosphorylation of I.kappa.B. It has been found by the present inventors that compounds of general formula I are effective as inhibitors of IKK.beta. in particular.

Detail Description Paragraph - DETX:

[0065] Furthermore, **kinase** profiling against about 40 different **kinases** has established that compounds of formula I are surprisingly selective for **IKK** and show little or no reactivity against the other **kinases** tested. This suggests a specific and unexpected effect of compounds of formula I rather than a general toxic effect.

Detail Description Paragraph - DETX:

[0114] In vitro **kinase** assay: THP-1 cells (1.times.10.sup.7 cells) were stimulated with 1 .mu.g/ml LPS for the indicated time periods and whole cell extracts were prepared as described above. **IKK** was immunoprecipitated with an IKK.alpha./.beta. antibody (raised against amino acid 470-755 of IKK.beta. of **human** origin but partially cross-reactive with IKK.alpha. as detected by Western Blotting (Santa Cruz Biotechnology Inc.). Compound A was diluted in **kinase** buffer (25 mM HEPES pH 7.5, 10 mM magnesium acetate, 50 .mu.M ATP) at the appropriate concentrations. The compound was added to the beads for 30 min. at 30.degree. C. The **kinase** reaction was started by adding 2.5 .mu.g of

the GST-I.kappa.B.alpha. substrate (Santa Cruz Biotechnology Inc.) together with 2 .mu.Ci .gamma.-.sup.32P-ATP (Amersham Pharmacia Biotech) per sample. The reaction was allowed to proceed for 30 min. at 30.degree. C., and the **IKK** activity was measured by the amount of radioactive-labelled GST-I.kappa.B.alpha. bands, quantified by the STORM860 Phospho-Imager.

Detail Description Paragraph - DETX:

[0115] A general problem with the **kinase** assay is prestimulation of the **kinase**. To achieve a value for the zero-point, the LPS-activated **IKK** mixture was incubated with 20 .mu.M myricetin (Sigma-Aldrich), which is a known IKK.beta. inhibitor (S. H. Tsai et al., J. Cell Biochem. 74, 1999, pp. 606-615). This value was subtracted from the all other values. All data are expressed as percentage of the activity of the LPS-activated IKK.beta. **kinase** treated with 0.1% DMSO.

Detail Description Paragraph - DETX:

[0116] **IKK** activity: Upon cellular activation by extracellular stimuli, I.kappa.B proteins are phosphorylated by a large I.kappa.B **kinase** complex. An in vitro **IKK** activity assay was established to evaluate a possible effect of compound A on the **IKK** activity. The THP-1 cells were stimulated with 1 .mu.g/ml LPS for 12 min, and then the cells were lysed and immunoprecipitated by an **IKK** antibody. The purified **IKK** was then pretreated with various concentrations of compound A ranging from 10.sup.-11 to 10.sup.-5 M for 30 min. prior to the **IKK** activity assay (FIG. 4). A "chemical zero-point" was introduced by treating the LPS-activated **IKK** with the IKK.beta. inhibitor myricetin (20 .mu.M) (S. H. Tsai et al., supra) to overcome the problem with a prestimulated **kinase**. A clear dose-response was observed as illustrated by the decrease of GST-I.kappa.B.alpha. phosphorylation In the compound A-treated samples. Four independent experiments were performed and the results are summarised on the plot (FIG. 4). The IC.sub.50 values range from 0.9 nM to 70 nM with a mean IC.sub.50 value of 8 nM.

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TITLE: Nod2 nucleic acids and proteins

PUBLICATION-DATE: December 26, 2002

INVENTOR-INFORMATION:

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DATE FILED: October 26, 2001

RELATED-US-APPL-DATA:

non-provisional-of-provisional 60244266 20001030 US
non-provisional-of-provisional 60286316 20010425 US

US-CL-CURRENT: 435/6

ABSTRACT:

The present invention relates to intracellular signalling molecules, in particular the Nod2 protein and nucleic acids encoding the Nod2 protein. The present invention provides isolated nucleotide sequence encoding Nod2, isolated Nod2 peptides, antibodies that specifically bind Nod2, methods for the detection of Nod2, and methods for screening compounds for the ability to alter Nod2 associated signal transduction. The present invention also provides Nod2 variant alleles. The present invention further provides methods of identifying individuals at increased risk of developing Crohn's disease.

[0001] This application claims priority to U.S. provisional patent applications serial Nos. 60/244,266 and 60/286,316, each of which is herein incorporated by reference in its entirety. This patent application was supported in part by grant CA-64556 from the National Institutes of Health. The government has certain rights in the invention.

---------- KWIC ---------

Detail Description Paragraph - DETX:

[0416] In the experimental disclosure which follows, the following abbreviations apply: eq (equivalents); M (Molar); .mu.M (micromolar); N (Normal); mol (moles); mmol (millimoles); .mu.mol (micromoles); nmol (nanomoles); g (grams); mg (milligrams); .mu.g (micrograms); ng (nanograms); 1 or L (liters); ml (milliliters); .mu.l (microliters); cm (centimeters); mm (millimeters); .mu.m (micrometers); nm (nanometers); .degree. C. (degrees Centigrade); U (units), mU (milliunits); min. (minutes); sec. (seconds); % (percent); kb (kilobase); bp (base pair); PCR (polymerase chain reaction); BSA (bovine serum albumin); Fisher (Fisher Scientific, Pittsburgh, Pa.); Sigma (Sigma Chemical Co., St. Louis, Mo.); Promega (Promega Corp., Madison, Wis.); Perkin-Elmer (Perkin-Elmer/Applied Biosystems, Foster City, Calif.); Boehringer Mannheim (Boehringer Mannheim, Corp., Indianapolis, Ind.); Clonetech (Clonetech, Palo Alto, Calif.); Qiagen (Qiagen, Santa Clarita, Calif.); Stratagene (Stratagene Inc., La Jolla, Calif.); National Biosciences (National Biosciences Inc, Plymouth Minn.) and NEB (New England Biolabs, Beverly, Mass.), CARD (caspase-recruitment domain); EST (expressed sequence tag); HA (hemagglutinin); I.kappa.B (inhibitor of NF-.kappa.B); **IKK** (I.kappa.b **kinase**); LRRs (leucine-rich repeats); NBD (nucleotide-binding domain); NF-.kappa.B (nuclear factor .kappa.B); TNF.alpha. (tumor necrosis factor .alpha.); wt (wild-type); Ab (antibody); IL-1 (interleukin 1); IL-1R (IL-1 receptor); LPS (lipopolysaccharide); LTA (lipoteichoic acid); PGN (peptidoglycan); SBLP (synthetic bacterial lipoprotein); and TLR (Toll-like receptor).

Detail Description Paragraph - DETX:

[0425] The Nod2 cDNA was cloned into pcDNA3-HA and pcDNA3-Fpk3-Myc (Inohara et al., [2000], supra). Deletion and site-directed mutants of Nod2 (129-1040, A125-214, 1-125, 1-301, 1-744, 265-1040, 126-301, 265744, 744-1040, K305R, 1-744K305R) were constructed by a PCR method and cloned into pcDNA3-HA and pcDNA3-Fpk3-Myc (Inohara et al., [2000], supra). The authenticity of all constructs was confirmed by sequencing. pcDNA3-Flag-RICK, pcDNA3-Flag-RICK(1-374), pcDNA3-Flag-RICK(374-540), pcDNA3-Myc-RICK(406-540), pcDNA3-Myc-RIP(558-671), pRK7-Flag-IKK.alpha., pRK7-FlagIKK.alpha.-K44A, RSVMad-3MSS(I.kappa.-B.alpha.-S32A/S36A), pRK7-Flag-IKK.beta., pRK7-Flag-IKK.beta.-K44A, and pcDNA3-Flag-IKK.gamma. (134-419) have been described previously (Inohara et al., supra, 10). The expression plasmids pcDNA3-Nod1-Flag, pcDNA3-Nod1 (1-648)-Flag, pcDNA3-Flag-**IKKi**, pcDNA3CIPER-Flag, pCMV-ILIR, pCMV-TLR4-Flag, pcDNA3-Flag-RIP, pcDNA3-MyD88 DN (amino acids 1-109), pcDNA3-CD14, pCMV-MD2-FLAG and pcDNA3-.beta.-gal have also been described previously (Inohara et al., [1999], Supra; Inohara et al., [1999], Supra; Inohara et al., [2000], supra; Shimada et al., Int. Immunol., 11:1357-1362 [1999]; Huang et al., PNAS, 94:12829-12832 [1997]; Medzhitov et al., Mol. Cell, 2:253-258 [1998]; Hsu et al., Immunity, 4:387-396 [1996]). To construct the expression plasmid producing C-terminally HA-tagged mature interleukin-1 P (IL1.beta.), pcDNA3-mIL1.beta.-HA, the mature region of **mouse** IL1.beta. was amplified by PCR and inserted into pcDNA3-HA-pro which contains the signal sequence of protrypsin and the HA tag.

Detail Description Paragraph - DETX:

[0437] This example demonstrates that NF-.kappa.B activation induced by Nod2 requires IKK.gamma. and is inhibited by dominant negative forms of **IKKs** and RICK. A main pathway of NF-.kappa.B activation is mediated by I.kappa.B **kinases (IKKS)** resulting in I.kappa.B phosporylation and release of cytoplamic NF-.kappa.B (Karin, J. Biol. Chem. 274: 27339-27342 [1999]). To determine whether Nod2 activates an **IKK**-dependent pathway, Nod2 was co-expressed with mutant forms of IKK.alpha., IKK.gamma., and I.kappa.B.alpha. that have been shown to act as dominant inhibitors of their corresponding endogenous counterparts and/or the **IKK** complex (Karin, supra). In addition, a truncated mutant of IKK.gamma./Nemo (residues 134-419) was used that is defective in IKK.alpha. and IKK.beta. binding and acts as an inhibitor of NF-.kappa.B activation induced by RIP and RICK (Inohara et al., [2000], supra). The NF-.kappa.B activity induced by Nod2 as well as that induced by TNF.alpha. stimulation were greatly inhibited by mutant IKK.alpha., IKK.gamma., IKK.gamma., and I.kappa.B.alpha. (FIG. 5A). Because RICK has been shown to serve as a downstream target of Nod1 (Bertin et al., supra, Inohara et al., [1999] supra, Inohara et al., [2000], supra), a truncated form of RICK containing its CARD (residues 406-540) that acts as a dominant inhibitor of Nod1 activity (Bertin et al., supra) was used to test whether NF-.kappa.B activation induced by Nod2 is similarly inhibited by this RICK mutant. NF-.kappa.B activation induced by Nod2 was inhibited by mutant RICK but not by a mutant form of RIP that expresses its death effector domain (FIG. 5A). The inhibition by the CARD of RICK was specific in that it did not interfere with ability of TNF.alpha. to induce NF-.kappa.B, an activity that was inhibited by the RIP mutant (FIG. 5A). To verify that Nod2 acts upstream of the **IKK** complex to activate NF-.kappa.B, we tested the ability of Nod2 to activate NF-.kappa.B in parental Ratl fibroblasts and 5R cells, a Ratl derivative cell line that is defective in IKK.gamma., an essential subunit of the **IKKs** (Yamaoka et al., supra). Nod2, as well as Nod1 and TNF.alpha. oc, induced NF-.kappa.B activity in parental Ratl cells but not in IKK.gamma.-deficient 5R cells (FIG. 5B). As a control, expression of IKK.beta., which functions downstream of IKK.gamma., induced NF-.kappa.B activation in both Ratl and 5R cell lines (FIG. 5B). These results indicate that Nod2 acts through IKK.gamma./IKK/IKK.beta. to activate NF-.kappa.B.

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PGPUB-FILING-TYPE: new

DOCUMENT-IDENTIFIER: US 20020156000 A1

TITLE: Anti-inflammatory compounds and uses thereof

PUBLICATION-DATE: October 24, 2002

INVENTOR-INFORMATION:

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DATE FILED: May 2, 2001

RELATED-US-APPL-DATA:

child 09847940 A1 20010502 parent continuation-in-part-of 09643260 20000822 US PENDING non-provisional-of-provisional 60201261 20000502 US

US-CL-CURRENT: 514/12

ABSTRACT:

The present invention provides anti-inflammatory compounds, pharmaceutical compositions thereof, and methods of use thereof for treating inflammatory disorders. The present invention also provides methods of identifying anti-inflammatory compounds and methods of inhibiting NF-.kappa.B-dependent target gene expression in a cell.

RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application Serial No. 60/201,261 filed May 2, 2000 and to U.S. patent application Ser. No. 09/643,260 filed Aug. 22, 2000, the entire contents of each of which are incorporated herein by reference.

---------- KWIC ---------

Summary of Invention Paragraph - BSTX:

[0004] NF-.kappa.B is a transcription factor which mediates extracellular signals responsible for induction of genes involved in pro-inflammatory responses (Baltimore et al., (1998) U.S. Pat. No. 5,804,374). NF-.kappa.B is

anchored in the cytoplasm of most non-stimulated cells by a non-covalent interaction with one of several inhibitory proteins known as I.kappa.Bs (May & Ghosh, (1997) Semin. Cancer. Biol. 8, 63-73; May & Ghosh, (1998) Immunol. Today 19, 80-88; Ghosh et al., (1998) Annu. Rev. Immunol. 16, 225-260). Cellular stimuli associated with pro-inflammatory responses such as TNF.alpha., activate **kinases,** which in turn activate NF-.kappa.B by phosphorylating I.kappa.Bs. The **kinases** that phosphorylate I.kappa.Bs are called I.kappa.B **kinases (IKKs)**.

Summary of Invention Paragraph - BSTX:

[0006] The identification and characterization of **kinases** that phosphorylate I.kappa.Bs has led to a better understanding of signaling pathways involving NF-.kappa.B activation. Several different subtypes of **IKK** have been identified thus far. IKK.alpha. was initially identified as an I.kappa.B **kinase** induced by TNF.alpha. stimulation in HeLa cells (DiDonato et al., (1997) Nature 388, 548-554). Another I.kappa.B **kinase** homologous to IKK.alpha. was identified, termed IKK.beta. and determined to be the major I.kappa.B **kinase** induced following TNF.alpha. stimulation (Takeda et al., (1999) Science 284, 313-316; Hu et al., (1999) Science 284, 316-320; Li et al., (1999) Science 284, 321-325; Pot et al., (2000) U.S. Pat. No. 6,030,834; Woronicz & Goeddel (1999) U.S. Pat. No. 5,939,302). IKK.alpha. and IKK.beta. have an overall homology of 52% and a 65% homology in the **kinase** domain (Zandi et al., (1997) Cell 91, 243-252).

Summary of Invention Paragraph - BSTX:

[0007] I.kappa.B protein **kinases (IKKs)** phosphorylate I.kappa.Bs at specific serine residues. For example, they specifically phosphorylate serines 32 and 36 of I.kappa.B.alpha. (Traenckner et al., (1995) EMBO J. 14, 2876-2883; DiDonato et al., (1996) Mol. Cell. Biol. 16, 1295-1304). Phosphorylation of both sites is required to efficiently target I.kappa.B.alpha. for degradation. Furthermore, activation of IKK.alpha. and IKK.beta. is usually in response to NF-.kappa.B activating agents and mutant IKK.alpha. and IKK.beta., which are catalytically inactive, can be used to block NF-.kappa.B stimulation by cytokines such as TNF.alpha. and IL-1 (Rgnier et al., (1997) Cell 90, 373-383; Delhase et al., (1999) Science 284, 309-313). I.kappa.B protein **kinases** are therefore essential in the regulation of NF-.kappa.B activation processes.

Summary of Invention Paragraph - BSTX:

[0008] IKK.alpha. and IKK.beta. have distinct structural motifs including an amino terminal serine-threonine **kinase** domain separated from a carboxyl proximal helix-loop-helix (H-L-H) domain by a leucine zipper domain. These structural characteristics are unlike other **kinases,** and the non-catalytic domains are thought to be involved in protein-protein interactions. Proteins which bind to **IKKs** may therefore be capable of regulating the activity of NF-.kappa.B (Marcu et al., (1999) U.S. Pat. No. 5,972,655) and potentially regulating downstream events such as induction of NF-.kappa.B. For instance,

NEMO (NF-.kappa.B Essential Modulator) is a protein which has been identified to bind to **IKKs** and facilitate **kinase** activity (Yamaoke et al., (1998) Cell 93, 1231-1240; Rothwarf et al., (1998) Nature 395, 287-300; Mercurio et al., (1999) Mol. Cell. Biol. 19, 1526-1538; Haraj & Sun, (1999) J. Biol. Chem. 274, 22911-22914; Jin & Jeang, (1999) J. Biomed. Sci. 6, 115-120).

Brief Description of Drawings Paragraph - DRTX:

[0027] FIG. 4 depicts results from experiments indicating that association of NEMO with IKK.beta. and IKK.alpha. reveals the NEMO binding domain (NBD) to be six COOH-terminal amino acids. (A) COS cells transiently transfected with vector alone, FLAG-tagged IKK.alpha. or IKK.beta. (1 .mu.g/well) or xpress-tagged NEMO (1 .mu.g/well) to a total DNA concentration of 2 .mu.g/well as indicated. Following lysis, immunoprecipitations (IP) were performed using anti-FLAG (M2) and the contents of precipitates visualized by immunoblotting (IB) with either anti-FLAG (M2) or anti-xpress. A portion of pre-IP lysate was immunoblotted with anti-xpress to ensure equivalent levels of NEMO expression in transfected cells. (B) Wild-type IKK.alpha. and IKK.alpha.-(1-737) were expressed and labeled (input) and used for GST pull-down using GST or GST-NEMO. (C) Full length cDNA encoding **human IKKi** was obtained by RT-PCR from HeLa cell mRNA using the Expand.TM. Long Template PCR System (Boehringer Mannheim), the forward primer (5'-CTAGTCGAATTCACCATGCAGAGCACAGCCAATTAC) (SEQ ID NO: 22) and
the reverse primer (3'-CTAGTCTCTAGATTAGACATCAGGAGGTGCTGG) (SEQ ID NO: 23) and
cloned into the EcoRI and Xbal sites of pcDNA-3. GST pull-down analysis was performed using [.sup.35S]-methionine-labeled IKK.alpha., IKK.beta. and **IKKi**. (D) A deletion mutant of IKK.beta. lacking the NBD (del.NBD) was [.sup.35S]-methionine-labeled (input) and used for GST pull down analysis. (E) A Fauchere-Pliska hydrophobicity plot of the COOH-terminus (N721-S756) of **human** IKK.beta. was generated using MacVector.quadrature. (version 6.5.3) software. The NBD (L737-L742) is boxed. (F) COS cells were transfected for forty-eight hours with a total of 2 .mu.g DNA/well of either vector alone, vector plus NEMO-FLAG or NEMO-FLAG plus xpress-tagged versions of IKK.beta.-(L-744) containing point mutations within the NBD as indicated. Following lysis and immunoprecipitation using anti-FLAG (M2), immunoblot analysis was performed with either anti-FLAG or anti-xpress. The level of expressed protein in pre-IP lysate was determined by immunoblotting with anti-xpress (lower panel). (G) HeLa cells were transiently transfected for forty-eight hours with the indicated constructs together with pBIIX-luciferase and NF.kappa.B activity in lysate was measured by luciferase assay.

Detail Description Paragraph - DETX:

[0048] As used herein, the term "I.kappa.B-**kinase**" or "I.kappa.B protein **kinase**" or "I.kappa.B-**kinase** complex" or "I.kappa.B protein **kinase** complex" or "**IKK**" refers to a **kinase** that phosphorylates I.kappa.Bs.

Detail Description Paragraph - DETX:

[0050] As used herein, the term "NEMO" (NF-.kappa.B Essential Modulator), "IKK.gamma." or "**IKKAP**" refers to the protein which binds to **IKKs** and facilitates **kinase** activity.

Detail Description Paragraph - DETX:

[0169] These results demonstrate that basal auto-phosphorylation and **kinase** activity of IKK.beta. is dependent on the ability of NEMO to associate with the **kinase**. One explanation for these observations may be that NEMO recruits a phosphatase to the **IKK**-complex that regulates basal IKK.beta. function by targeting the serine-rich region of the COOH-terminus. Inability to bind NEMO therefore prevents phosphatase recruitment and causes increased phosphorylation within this region.

Detail Description Paragraph - DETX:

[0175] Further evidence that this short COOH-terminal sequence constitutes the NEMO-interaction domain of the **IKKs** was obtained when we tested the ability of the recently described **IKK**-related **kinase IKKi** (Shimada et al., (1999) Int. Immunol. 11, 1357-1362) to interact with NEMO. Sequence comparison with IKK.alpha. and IKK.beta. (Shimada et al., (1999) Int. Immunol. 11, 1357-1362; Woronicz et al., (1997) Science 278, 866-869; Zandi et al., (1997) Cell 91, 243-52; Mercurio et al., (1997) Science 278, 860-866; DiDonato et al., (1997) Nature 388, 548-554; Regnier et al., (1997) Cell 90, 373-383) reveals that **IKKi** does not contain the .alpha.2-region in its COOH-terminus (Shimada et al., (1999) Int. Immunol. 11, 1357- 1362) and consistent with this being the NEMO binding domain we found that **IKKi** does not interact with GST-NEMO in pull down assays (FIG. 4C). This finding indicates that NEMO is not required for the functional activity of **IKKi** and this is supported by the inability of **IKKi** to respond to signals induced by either TNF.alpha. or IL-1.beta. (Shimada et al., (1999) Int. Immunol. 11, 1357-1362).

Detail Description Paragraph - DETX:

[0183] The importance of the present invention can be viewed on two levels. First, Applicants have identified the structural requirements for the association of NEMO with the **IKKs** and found that association with IKK.beta. is dependent on three amino acids (D738, W739 and W741) within the NBD. Furthermore, NEMO not only functions in the activation of IKK.beta. but it also has a critical role in suppressing the intrinsic, basal activity of the **IKK** complex. The second level of importance is the obvious clinical use for drugs targeting the NBD. Applicants have demonstrated that a cell-permeable peptide encompassing the NBD is able to not only inhibit TNF.alpha.-induced NF-.kappa.B activation but also reduce expression of E-selectin, an NF-.kappa.B-dependent target gene, in primary **human** endothelial cells. The NBD is only six amino acids long, and therefore it is within the ability of one skilled in the art to design peptido-mimetic compounds that disrupt the core **IKK** complex. Since the effect of disrupting the complex is to increase the basal activity of the **IKK**, treatment with an NBD-targeting compound can avoid

issues of toxicity, e.g., due to hepatocyte apoptosis, that might arise from administering drugs that completely abolish the activity of NF-.kappa.B. Hence, identification of the NBD is a means for the development of novel anti-inflammatory drugs that prevent activating signals from reaching the **IKK** complex, yet maintain a low level of NF-.kappa.B activity and avoid potential toxic side-effects.

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ABSTRACT:

The present invention provides novel methods for ligand discovery. The inventive methods rely on a process termed "tethering" where potential ligands are covalently bonded or "tethered" to a target and subsequently identified.

[0001] This application is a continuation-in-part of U.S. Ser. No. 09/981,547 filed Oct. 17, 2001 which is a divisional of U.S. Ser. No. 09/105,372 filed Jun. 26, 1998, and is a continuation-in-part of U.S. Ser. No. 09/990,421 filed Nov. 21, 2001 which asserts priority to U.S. Provisional Application No. 60/252,294 filed Nov. 21, 2000, all of which are incorporated herein by reference.

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Detail Description Paragraph - DETX:

[0087] Preferred protein targets include: cell surface and soluble receptor

proteins, such as lymphocyte cell surface receptors; enzymes; proteases (e.g., aspartyl, cysteine, metallo, and serine); steroid receptors; nuclear proteins; allosteric enzymes; clotting factors; **kinases** (serine/threonine **kinases** and tyrosine **kinases**); phosphatases (serine/threonine, tyrosine, and dual specificity phosphatases, especially PTP-1B, TC-PTP and LAR); thymidylate synthase; bacterial enzymes, fungal enzymes and viral enzymes (especially those associated with HIV, influenza, rhinovirus and RSV); signal transduction molecules; transcription factors; proteins or enzymes associated with DNA and/or RNA synthesis or degradation; immunoglobulins; hormones; and receptors for various cytokines. Illustrative examples of receptors include for example, erythropoietin (EPO), granulocyte colony stimulating (G-CSF) receptor, granulocyte macrophage colony stimulating (GM-CSF) receptor, thrombopoietin (TPO), interleukins, e.g. IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-11, IL-12, growth hormone, prolactin, **human** placental lactogen (LPL), CNTF, oncostatin, RANTES, MIPb, IL-8, insulin, insulin-like growth factor 1 (IGF-1), epidermal growth factor (EGF), heregulin-a and heregulin-b, vascular endothelial growth factor (VEGF), placental growth factor (PLGF), tissue growth factors (TGF-.alpha. and TGF-.beta.), and nerve growth factor (NGF). Other targets include various neurotrophins and their ligands, other hormones and receptors such as, bone morphogenic factors, follicle stimulating hormone (FSH), and luteinizing hormone (LH), CD40 ligand, apoptosis factor-1 and -2 (AP-1 and AP-2), p53, bax/bcl2, mdm2, caspases (1, 3, 8 and 9), cathepsins, IL-1/IL-1 receptor, BACE, HIV integrase, PDE IV, Hepatitis C helicase, Hepatitis C protease, rhinovirus protease, tryptase, cPLA (cytosolic Phospholipase A2), CDK4, c-jun **kinase,** adaptors such as Grb2, GSK-3, AKT, MEKK-1, PAK-1, raf, TRAF's 1-6, Tie2, ErbB 1 and 2, FGF, PDGF, PARP, CD2, C5a receptor, CD4, CD26, CD3, TGF-alpha, NF-kB, **IKK** beta, STAT 6, Neurokinnin-1, CD45, Cdc25A, SHIP-2, **human** p53, bax/bc12, IgE/IgER, ZAP-70, Ick, syk, ITK/BTK, TACE, Cathepsin S, K and F, CD11a, LFA/ICAM, VLA-4, CD28/B7, CTLA4, TNF alpha and beta, (and the p55 and p75 TNF receptors), CD40L, p38 map **kinase,** IL-2, IL-4, Il-13, IL-15, Rac 2, PKC theta, IL-8, TAK-1, jnk, **IKK2** and IL-18.

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ABSTRACT:

Compositions and methods are provided for treating NF-.kappa.B-related conditions. In particular, the invention provides a stimulus-inducible IKK signalsome, and components and variants thereof. An IKK signalsome or component thereof may be used, for example, to identify antibodies and other modulating agents that inhibit or activate signal transduction via the NF-.kappa.B cascade. IKK signalsome, components thereof and/or modulating agents may also be used for the treatment of diseases associated with NF-.kappa.B activation.

CROSS-REFERENCE TO PRIOR APPLICATION

[0001] This application is a continuation-in-part of U.S. patent application Ser. No. 08/697,393, filed Aug. 26, 1996.

---------- KWIC ---------

Summary of Invention Paragraph - BSTX:

[0002] The present invention relates generally to compositions and methods useful for the study of cascades leading to the activation of nuclear factor .kappa.B (NF-.kappa.B) and for treating diseases associated with such pathways. The invention is more particularly related to a stimulus-inducible I.kappa.B **kinase (IKK)** signalsome, component I.kappa.B **kinases** and variants of such **kinases**. The present invention is also related to the use of a stimulus-inducible **IKK** signalsome or I.kappa.B **kinase** to identify antibodies and other agents that inhibit or activate signal transduction via the NF-.kappa.B pathway.

Summary of Invention Paragraph - BSTX:

[0017] In further aspects, the present invention provides methods for modulating NF-.kappa.B activity in a patient, comprising administering to a patient an agent that modulates I.kappa.B **kinase** activity in combination with a pharmaceutically acceptable carrier. Methods are also provided for treating a patient afflicted with a disorder associated with the activation of **IKK** signalsome, comprising administering to a patient a therapeutically effective amount of an agent that modulates I.kappa.B **kinase** activity in combination with a pharmaceutically acceptable carrier.

Summary of Invention Paragraph - BSTX:

[0020] In a further aspect, the present invention provides a method for identifying an upstream **kinase** in the NF-.kappa.B signal transduction cascade, comprising evaluating the ability of a candidate upstream **kinase** to phosphorylate an **IKK** signalsome, a component thereof or a variant of such a component.

Summary of Invention Paragraph - BSTX:

[0022] In yet another aspect, a method is provided for preparing an **IKK** signalsome from a biological sample, comprising: (a) separating a biological sample into two or more fractions; and (b) monitoring I.kappa.B **kinase** activity in the fractions.

Brief Description of Drawings Paragraph - DRTX:

[0033] FIGS. 8A-8C are autoradiograms depicting the results of immunoblot analyses. In FIG. 8A, the upper panel presents a time course for the induction of signalsome activity. Anti MKP-1 immune precipitates from extracts of HeLa S3 cells stimulated with TNF.alpha. (20 ng/ml) for the indicated times, were assayed for **IKK** signalsome activity by standard immune complex **kinase** assays. 4 .mu.g of either GST I.kappa.B.alpha. 1-54 WT (wildtype) or the GST I.kappa.B.alpha. 1-54 S32/36 to T mutant (S>T) were used as the substrates.

In the lower panel, HeLa cell extracts prepared as described in the upper panel were examined by western blot analysis for I.kappa.B.alpha. degradation. I.kappa.B.alpha. supershifting phosphorylation can be seen after 3 and 5 minutes of stimulation followed by the disappearance of I.kappa.B.alpha..

Brief Description of Drawings Paragraph - DRTX:

[0038] FIG. 10 is an autoradiogram showing the results of a western blot analysis of the level of ubiquitin within a stimulus-inducible IkB **kinase** complex. Lane 1 shows the detection of 100 ng ubiquitin, Lane 2 shows 10 ng ubiquitin and Lane 3 shows 3.4 .mu.g of **IKK** signalsome purified through the phenyl superose step (sufficient quantities for 10 **kinase** reactions). The position of ubiquitin is shown by the arrow on the left.

Brief Description of Drawings Paragraph - DRTX:

[0039] FIG. 11A illustrates a procedure for purification of the **IKK** signalsome. A whole cell extract was prepared from TNF.alpha.-stimulated (20 ng/ml, 7 minute induction) HeLa S3 cells (1.2 g total protein). The **IKK** signalsome was then immunoprecipitated from the extract using anti-MKP-1 antibodies, washed with buffer containing 3.5 M urea and eluted overnight at 4.degree. C. in the presence of excess MKP-1 specific peptide. Eluted **IKK** signalsome was then fractionated on a Mono Q column, I.kappa.B **kinase** active fractions were pooled, concentrated and subjected to preparative SDS-PAGE. Individual protein bands were excised and submitted for peptide sequencing.

Brief Description of Drawings Paragraph - DRTX:

[0042] FIG. 13A illustrates the amino acid sequence of **IKK-1 and IKK-2**. Symbols: arrows, boundaries of the **kinase** domain; underlined, peptide sequences identified by nanoelectrospray mass spectrometry; asterisks, indicates leucines comprising the leucine zipper motif; bold face, indicate amino acid identities conserved between **IKK-1 and IKK-2**; highlighted box, Helix-loop-helix domain; dashes, a gap inserted to optimize alignment.

Brief Description of Drawings Paragraph - DRTX:

[0043] FIG. 13B is an autoradiogram depicting the results of Northern blot analysis of **IKK-2** mRNA in adult **human** tissue. The source of the tissue is indicated at the top. Probes spanning the coding region of **human IKK-2** and .beta.-actin cDNA were used and are indicated to the left. Molecular weight standards are indicated to the right.

Brief Description of Drawings Paragraph - DRTX:

[0044] FIG. 14A is an autoradiogram depicting the results of **kinase** assays using **IKK-1 and IKK-2**. **IKK-1 and IKK-2** were immunoprecipitated from rabbit

reticulocyte lysates phosphorylate I.kappa.B.alpha. and I.kappa.B.beta.. Either HA-tagged **IKK-1** (lane 1) or Flag-tagged **IKK-2** (lane 2) were translated in rabbit reticulocyte lysates, immunoprecipitated, and examined for their ability to phosphorylate GST I.kappa.B.alpha. 1-54 WT and GST I.kappa.B.beta. 1-44 as indicated by an arrow to the left. **IKK-1** (lane 1) undergoes significant autophosphorylation in contrast to **IKK-2** (lane 2) which is identified only with longer exposure times.

Brief Description of Drawings Paragraph - DRTX:

[0045] FIGS. 14B and 14C are micrographs illustrating the results of assays to evaluate the ability of **kinase**-inactive mutants of **IKK-1 and IKK-2** to inhibit RelA translocation in TNF.alpha.-stimulated HeLa cells. HeLa cells were transiently transfected with either HA-tagged **IKK-1** K44 to M mutant (14B) or Flag-tagged **IKK-2** K44 to M mutant (14C) expression vectors. 36 hours post-transfection cells were either not stimulated (Unstim) or TNF.alpha.-stimulated (20 ng/ml) for 30 min (TNF.alpha.), as indicated to the right of the figure. Cells were then subjected to immunofluorescence staining using anti-HA of anti-Flag antibodies to visualize expression of **IKK-1** K44 to M or **IKK-2** K44 to M, respectively. Stimulus-dependent translocation of Rel A was monitored using anti-Rel A antibodies. Antibodies used are indicated to the top of the figure. **IKK** mutant transfected is indicated to the left of the figure.

Detail Description Paragraph - DETX:

[0047] As noted above, the present invention is generally directed to compositions and methods for modulating (i.e., stimulating or inhibiting) signal transduction leading to NF-.kappa.B activation. In particular, the present invention is directed to compositions comprising an I.kappa.B **kinase (IKK)** signalsome (also referred to herein as a "stimulus-inducible I.kappa.B **kinase** complex" or "I.kappa.B **kinase** complex") that is capable of stimulus-dependent phosphorylation of I.kappa.B.alpha. and I.kappa.B.beta. on the two N-terminal serine residues critical for the subsequent ubiquitination and degradation in vivo. Such stimulus-dependent phosphorylation may be achieved without the addition of exogenous cofactors. In particular, an **IKK** signalsome specifically phosphorylates I.kappa.B.alpha. (SEQ ID NO:1) at residues S32 and S36 and phosphorylates I.kappa.B.beta. (SEQ ID NO:2) at residues S19 and S23. The present invention also encompasses compositions that contain one or more components of such an **IKK** signalsome, or variants of such components. Preferred components, referred to herein as "**IKK** signalsome **kinases**" "I.kappa.B **kinases" or IKKs) are kinases** that, when incorporated into an **IKK** signalsome, are capable of phosphorylating I.kappa.B.alpha. at S32 and S36. Particularly preferred components are **IKK-1** (SEQ ID NO:10) and **IKK-2** (SEQ ID NO:9).

Detail Description Paragraph - DETX:

[0048] An **IKK** signalsome and/or I.kappa.B **kinase** may generally be used for

phosphorylating a substrate (i.e., an I.kappa.B, such as I.kappa.B.alpha., or a portion or variant thereof that can be phosphorylated at those residues that are phosphorylated in vivo) and for identifying modulators of I.kappa.B **kinase** activity. Such modulators and methods employing them for modulating I.kappa.B.alpha. **kinase** activity, in vivo and/or in vitro, are also encompassed by the present invention. In general, compositions that inhibit I.kappa.B **kinase** activity may inhibit I.kappa.B phosphorylation, or may inhibit the activation of an I.kappa.B **kinase** and/or **IKK** signalsome.

Detail Description Paragraph - DETX:

[0049] An **IKK** signalsome has several distinctive properties. Such a complex is stable (i.e., its components remain associated following purification as described herein) and has a high-molecular weight (about 500-700 kD, as determined by gel filtration chromatography). As shown in FIGS. 3 (A and B) and 4 (A and B), I.kappa.B **kinase** activity of an **IKK** signalsome is "stimulus-inducible" in that it is stimulated by TNF.alpha. (i.e., treatment of cells with TNF.alpha. results in increased I.kappa.B **kinase** activity and I.kappa.B degradation) and/or by one or more other inducers of NF-.kappa.B, such as IL-1, LPS, TPA, UV irradiation, antigens, viral proteins and stress-inducing agents. The kinetics of stimulation by TNF.alpha. correspond to those found in vivo. I.kappa.B **kinase** activity of an **IKK** signalsome is also specific for S32 and S36 of I.kappa.B.alpha.. As shown in FIGS. 5 (A and B) and 6 (A and B), an **IKK** signalsome is capable of phosphorylating a polypeptide having the N-terminal sequence of I.kappa.B.alpha. (GST-I.kappa.B.alpha.1-54; SEQ ID NO:3), but such phosphorylation cannot be detected in an I.kappa.B.alpha. derivative containing threonine substitutions at positions 32 and 36. In addition, I.kappa.B **kinase** activity is strongly inhibited by a doubly phosphorylated I.kappa.B.alpha. peptide (i.e., phosphorylated at S32 and S36), but not by an unrelated c-fos phosphopeptide that contains a single phosphothreonine. A further characteristic of an **IKK** signalsome is its ability to phosphorylate a substrate in vitro in a standard **kinase** buffer, without the addition of exogenous cofactors. Free ubiquitin is not detectable in preparations of **IKK** signalsome (see FIG. 10), even at very long exposures. Accordingly an **IKK** signalsome differs from the ubiquitin-dependent I.kappa.B.alpha. **kinase** activity described by Chen et al., Cell 84:853-62, 1996.

Detail Description Paragraph - DETX:

[0050] An **IKK** signalsome may be immunoprecipitated by antibodies raised against MKP-1 (MAP **kinase** phosphatase-1; Santa Cruz Biotechnology, Inc., Santa Cruz, Calif. #SC-1102), and its activity detected using an in vitro I.kappa.B.alpha. **kinase** assay. However, as discussed further below, MKP-1 does not appear to be a component of I.kappa.B **kinase** complex. The substrate specificity of the immunoprecipitated **IKK** signalsome is maintained (i.e., there is strong phosphorylation of wildtype GST-I.kappa.B.alpha. 1-54 (SEQ ID NO:3) and GST-I.kappa.B.beta. 1-44 (SEQ ID NO:4), and substantially no detectable phosphorylation of GST-I.kappa.B.alpha. 1-54 in which serines 32 and 36 are replaced by threonines (GST- I.kappa.B.alpha. S32/36 to T; SEQ ID NO:5) or

GST-I.kappa.B.beta. 1-44 in which serines 19 and 23 are replaced by alanines (GST-I.kappa.B.beta. 1-44 S19/23 to A; SEQ ID NO:6)).

Detail Description Paragraph - DETX:

[0051] An **IKK** signalsome may be isolated from **human** or other cells, and from any of a variety of tissues and/or cell types. For example, using standard protocols, cytoplasmic and/or nuclear/membrane extracts may be prepared from HeLa S3 cells following seven minutes induction with 30 ng/mL TNF.alpha.. The extracts may then be subjected to a series of chromatographic steps that includes Q Sepharose, gel filtration (HiLoad 16/60 Superdex 200), Mono Q, Phenyl Superose, gel filtration (Superdex 200 10/30) and Mono Q. This representative purification procedure is illustrated in FIG. 2, and results in highly enriched **IKK** signalsome (compare, for example, FIGS. 5A and 6A).

Detail Description Paragraph - DETX:

[0053] Throughout the fractionation, an in vitro **kinase** assay may be used to monitor the I.kappa.B **kinase** activity of the **IKK** signalsome. In such an assay, the ability of a fraction to phosphorylate an appropriate substrate (such as I.kappa.B.alpha. (SEQ ID NO:1) or a derivative or variant thereof) is evaluated by any of a variety of means that will be apparent to those of ordinary skill in the art. For example, a substrate may be combined with a chromatographic fraction in a protein **kinase** buffer containing .sup.32P .gamma.-ATP, phosphatase inhibitors and protease inhibitors. The mixture may be incubated for 30 minutes at 30.degree. C. The reaction may then be stopped by the addition of SDS sample buffer and analyzed using SDS-PAGE with subsequent autoradiography. Suitable substrates include full length I.kappa.B.alpha. (SEQ ID NO:1), polypeptides comprising the N-terminal 54 amino acids of I.kappa.B.alpha., full length I.kappa.B.beta. (SEQ ID NO:2) and polypeptides comprising the N-terminal 44 amino acids of I.kappa.B.beta.. Any of these substrates may be used with or without an N-terminal tag. One suitable substrate is a protein containing residues 1-54 of I.kappa.B.alpha. and an N-terminal GST tag (referred to herein as GST-I.kappa.B.alpha. 1-54; SEQ ID NO:3). To evaluate the specificity of an I.kappa.B **kinase** complex, I.kappa.B.alpha. mutants containing threonine or alanine residues at positions 32 and 36, and/or other modifications, may be employed.

Detail Description Paragraph - DETX:

[0056] Alternatively, partial sequences of the components may be obtained using standard biochemical purification and microsequencing techniques. For example, purified complex as described above may be run on an SDS-PAGE gel and individual bands may be isolated and subjected to protein microsequencing. DNA sequences encoding components may then be prepared by amplification from a suitable **human** cDNA library, using polymerase chain reaction (PCR) and methods well known to those of ordinary skill in the art. For example, an adapter-ligated cDNA library prepared from a cell line or tissue that expresses IKK signalsome (such as HeLa or Jurkat cells) may be screened using a degenerate 5' specific forward primer and an adapter-specific primer.

Degenerate oligonucleotides may also be used to screen a cDNA library, using methods well known to those of ordinary skill in the art. In addition, known proteins may be identified via Western blot analysis using specific antibodies.

Detail Description Paragraph - DETX:

[0057] Components of an **IKK** signalsome may also be identified by performing any of a variety of protein-protein interaction assays known to those of ordinary skill in the art. For example, a known component can be used as "bait" in standard two-hybrid screens to identify other regulatory molecules, which may include **IKK-1, IKK-2,** NF.kappa.B1, RelA, I.kappa.B.beta. and/or p70 S6 **kinase** (Kieran et al., Cell 62:1007-1018, 1990; Nolan et al., Cell 64:961-69, 1991; Thompson et al., Cell 80:573-82, 1995; Grove et al., Mol. Cell. Biol. 11:5541-50, 1991).

Detail Description Paragraph - DETX:

[0058] Particularly preferred components of **IKK** signalsome are I.kappa.B **kinases**. An I.kappa.B **kinase** may be identified based upon its ability to phosphorylate one or more I.kappa.B proteins, which may be readily determined using the representative **kinase** assays described herein. In general, an I.kappa.B **kinase** is incorporated into an **IKK** signalsome, as described herein, prior to performing such assays, since an I.kappa.B **kinase** that is not complex-associated may not display the same phosphorylation activity as complex-associated I.kappa.B **kinase**. As noted above, an I.kappa.B **kinase** within an **IKK** signalsome specifically phosphorylates I.kappa.B.alpha. at residues S32 and S36, and phosphorylates I.kappa.B.beta. at residues 19 and 23, in response to specific stimuli.

Detail Description Paragraph - DETX:

[0059] As noted above, **IKK-1 and IKK-2** are particularly preferred I.kappa.B **kinases**. **IKK-1 and IKK-2** may be prepared by pooling the fractions from the Mono Q column containing peak I.kappa.B **kinase** activity and subjecting the pooled fractions to preparative SDS gel electrophoresis. The intensity of two prominent protein bands of .about.85 and .about.87 kDa (indicated by silver stain in FIG. 11B as **IKK-1 and IKK-2** respectively) correlates with the profile of I.kappa.B **kinase** activity. The .about.85 kDa band, corresponding to **IKK-1,** has been identified, within the context of the present invention, as CHUK (conserved helix-loop-helix ubiquitous **kinase**; see Connely and Marcu, Cell. Mol. Biol. Res. 41:537-49,1995). The .about.87 kDa band contains **IKK-2**.

Detail Description Paragraph - DETX:

[0060] Sequence analysis reveals that **IKK-1 and IKK-2** are related protein serine **kinases** (51% identity) containing protein interaction motifs (FIG. 13A). Both **IKK-1 and IKK-2** contain the **kinase** domain at the N-terminus, and a leucine zipper motif and a helix-loop-helix motif in their C-terminal regions.

Northern analysis indicates that mRNAs encoding **IKK-2** are widely distributed in **human** tissues, with transcript sizes of .about.4.5 kb and 6 kb (FIG. 13B). The sequences of **IKK-1 and IKK-2** are also provided as SEQ ID NOs: 7 and 8, respectively.

Detail Description Paragraph - DETX:

[0061] It has been found, within the context of the present invention, that rabbit reticulocyte lysate immunoprecipitates that contain **IKK-1 or IKK-2** phosphorylate I.kappa.B.alpha. and I.kappa.B.beta. with the correct substrate specificity (see FIG. 14A). Altered versions of these **kinases** interfere with translocation of RelA to the nucleus of TNF.alpha.-stimulated HeLa cells. Accordingly, **IKK-1 and IKK-2** appear to control a significant early step of NF.kappa.B activation.

Detail Description Paragraph - DETX:

[0062] Other components of an **IKK** signalsome are also contemplated by the present invention. Such components may include, but are not limited to, upstream **kinases** such as MEKK-1 (Lee et al., Cell 88,:213-22, 1997; Hirano et al., J. Biol. Chem. 271:13234-38, 1996) or NIK (Malinin et al., Nature 385:540-44, 1997); adapter proteins that mediate an **IKK**-1:IKK-2 interaction; a component that crossreacts with anti-MKP-1; an inducible RelA **kinase**; and/or the E3 ubiquitin ligase that transfers multiubiquitin chains to phosphorylated I.kappa.B (Hershko and Ciechanover, Annu. Rev. Biochem. 61:761-807, 1992).

Detail Description Paragraph - DETX:

[0067] In one aspect of the present invention, an **IKK** signalsome and/or one or more components thereof may be used to identify modulating agents, which may be antibodies (e.g., monoclonal), polynucleotides or other drugs, that inhibit or stimulate signal transduction via the NF-.kappa.B cascade. Modulation includes the suppression or enhancement of NF-.kappa.B activity. Modulation may also include suppression or enhancement of I.kappa.B phosphorylation or the stimulation or inhibition of the ability of activated (i.e., phosphorylated) **IKK** signalsome to phosphorylate a substrate. Compositions that inhibit NF-.kappa.B activity by inhibiting I.kappa.B phosphorylation may include one or more agents that inhibit or block I.kappa.B.alpha. **kinase** activity, such as an antibody that neutralizes **IKK** signalsome, a competing peptide that represents the substrate binding domain of I.kappa.B **kinase** or a phosphorylation motif of I.kappa.B, an antisense polynucleotide or ribozyme that interferes with transcription and/or translation of I.kappa.B **kinase,** a molecule that inactivates **IKK** signalsome by binding to the complex, a molecule that binds to I.kappa.B and prevents phosphorylation by **IKK** signalsome or a molecule that prevents transfer of phosphate groups from the **kinase** to the substrate. Within certain embodiments, a modulating agent inhibits or enhances the expression or activity of **IKK-1** and/or **IKK-2**.

Detail Description Paragraph - DETX:

[0068] In general, modulating agents may be identified by combining a test compound with an **IKK** signalsome, I.kappa.B **kinase** or a polynucleotide encoding an I.kappa.B **kinase** in vitro or in vivo, and evaluating the effect of the test compound on the I.kappa.B **kinase** activity using, for example, a representative assay described herein. An increase or decrease in **kinase** activity can be measured by adding a radioactive compound, such as .sup.32P-ATP and observing radioactive incorporation into a suitable substrate for **IKK** signalsome, thereby determining whether the compound inhibits or stimulates **kinase** activity. Briefly, a candidate agent may be included in a reaction mixture containing compounds necessary for the **kinase** reaction (as described herein) and I.kappa.B substrate, along with **IKK** signalsome, I.kappa.B **kinase** or a polynucleotide encoding an I.kappa.B **kinase**. In general, a suitable amount of antibody or other agent for use in such an assay ranges from about 0.01 .mu.M to about 10 .mu.M. The effect of the agent on I.kappa.B **kinase** activity may then be evaluated by quantitating the incorporation of [.sup.32P]phosphate into an I.kappa.B such as I.kappa.B.alpha. (or a derivative or variant thereof), and comparing the level of incorporation with that achieved using I.kappa.B **kinase** without the addition of a candidate agent. Alternatively, the effect of a candidate modulating agent on transcription of an I.kappa.B **kinase** may be measured, for example, by Northern blot analysis or a promoter/reporter-based whole cell assay.

Detail Description Paragraph - DETX:

[0069] Alternatively, for assays in which the test compound is combined with an **IKK** signalsome, the effect on a different **IKK** signalsome activity may be assayed. For example, an **IKK** signalsome also displays p65 **kinase** activity and **IKK** phosphatase activity. Assays to evaluate the effect of a test compound on such activities may be performed using well known techniques. For example, assays for p65 **kinase** activity may generally be performed as described by Zhong et al., Cell 89:413-24, 1997. For phosphatase activity, an assay may generally be performed as described by Sullivan et al., J. Biomolecular Screening 2:19-24, 1997, using a recombinant phosphorylated I.kappa.B **kinase** as a substrate.

Detail Description Paragraph - DETX:

[0070] In another aspect of the present invention, **IKK** signalsome or I.kappa.B **kinase** may be used for phosphorylating an I.kappa.B such as I.kappa.B.alpha. (or a derivative or variant thereof) so as to render it a target for ubiquitination and subsequent degradation. I.kappa.B may be phosphorylated in vitro by incubating **IKK** signalsome or I.kappa.B **kinase** with I.kappa.B in a suitable buffer for 30 minutes at 30.degree. C. In general, about 0.01 .mu.g to about 9 .mu.g of I.kappa.B **kinase** complex is sufficient to phosphorylate from about 0.5 .mu.g to about 2 .mu.g of I.kappa.B. Phosphorylated substrate may then be purified by binding to GSH-sepharose and washing. The extent of substrate phosphorylation may generally be monitored by adding [.gamma.-.sup.32P]ATP to a test aliquot, and evaluating the level of substrate

phosphorylation as described herein.

Detail Description Paragraph - DETX:

[0071] An **IKK** signalsome, component thereof, modulating agent and/or polynucleotide encoding a component and/or modulating agent may also be used to modulate NF-.kappa.B activity in a patient. Such modulation may occur by any of a variety of mechanisms including, but not limited to, direct inhibition or enhancement of I.kappa.B phosphorylation using a component or modulating agent; or inhibiting upstream activators, such as NIK or MEK, with **IKK** signalsome or a component thereof. As used herein, a "patient" may be any mammal, including a **human,** and may be afflicted with a disease associated with I.kappa.B **kinase** activation and the NF-.kappa.B cascade, or may be free of detectable disease. Accordingly, the treatment may be of an existing disease or may be prophylactic. Diseases associated with the NF-.kappa.B cascade include inflammatory diseases, neurodegenerative diseases, autoimmune diseases, cancer and viral infection.

Detail Description Paragraph - DETX:

[0072] Treatment may include administration of an **IKK** signalsome, a component thereof and/or an agent which modulates I.kappa.B **kinase** activity. For administration to a patient, one or more such compounds are generally formulated as a pharmaceutical composition. A pharmaceutical composition may be a sterile aqueous or non-aqueous solution, suspension or emulsion, which additionally comprises a physiologically acceptable carrier (i.e., a non-toxic material that does not interfere with the activity of the active ingredient). Any suitable carrier known to those of ordinary skill in the art may be employed in the pharmaceutical compositions of the present invention. Representative carriers include physiological saline solutions, gelatin, water, alcohols, natural or synthetic oils, saccharide solutions, glycols, injectable organic esters such as ethyl oleate or a combination of such materials. Optionally, a pharmaceutical composition may additionally contain preservatives and/or other additives such as, for example, antimicrobial agents, anti-oxidants, chelating agents and/or inert gases, and/or other active ingredients.

Detail Description Paragraph - DETX:

[0079] In another aspect, the present invention provides methods for detecting the level of stimulus-inducible I.kappa.B **kinase** activity in a sample. The level of I.kappa.B **kinase** activity may generally be determined via an immunokinase assay, in which **IKK** signalsome is first immunoprecipitated with an antibody that binds to the complex. The immunoprecipitated material is then subjected to a **kinase** assay as described herein. Substrate specificity may be further evaluated as described herein to distinguish the activity of a stimulus-inducible I.kappa.B **kinase** complex from other **kinase** activities.

Detail Description Paragraph - DETX:

[0080] The present invention also provides methods for detecting the level of **IKK** signalsome, or a component thereof, in a sample. The amount of **IKK** signalsome, I.kappa.B **kinase** or nucleic acid encoding I.kappa.B **kinase,** may generally be determined using a reagent that binds to I.kappa.B **kinase,** or to DNA or RNA encoding a component thereof. To detect nucleic acid encoding a component, standard hybridization and/or PCR techniques may be employed using a nucleic acid probe or a PCR primer. Suitable probes and primers may be designed by those of ordinary skill in the art based on the component sequence. To detect **IKK** signalsome or a component thereof, the reagent is typically an antibody, which may be prepared as described below.

Detail Description Paragraph - DETX:

[0082] Antibodies encompassed by the present invention may be polyclonal or monoclonal, and may bind to **IKK** signalsome and/or one or more components thereof (e.g., **IKK-1** and/or **IKK-2**). Preferred antibodies are those antibodies that inhibit or block I.kappa.B **kinase** activity in vivo and within an in vitro assay, as described above. Other preferred antibodies are those that bind to one or more I.kappa.B proteins. As noted above, antibodies and other agents having a desired effect on I.kappa.B **kinase** activity may be administered to a patient (either prophylactically or for treatment of an existing disease) to modulate the phosphorylation of an I.kappa.B, such as I.kappa.B.alpha., in vivo.

Detail Description Paragraph - DETX:

[0086] In a related aspect of the present invention, kits for detecting the level of **IKK** signalsome, I.kappa.B **kinase,** nucleic acid encoding I.kappa.B **kinase** and/or I.kappa.B **kinase** activity in a sample are provided. Any of a variety of samples may be used in such assays, including eukaryotic cells, bacteria, viruses, extracts prepared from such organisms and fluids found within living organisms. In general, the kits of the present invention comprise one or more containers enclosing elements, such as reagents or buffers, to be used in the assay.

Detail Description Paragraph - DETX:

[0087] A kit for detecting the level of **IKK** signalsome, I.kappa.B **kinase** or nucleic acid encoding I.kappa.B **kinase** typically contains a reagent that binds to the compound of interest. To detect nucleic acid encoding I.kappa.B **kinase,** the reagent may be a nucleic acid probe or a PCR primer. To detect **IKK** signalsome or I.kappa.B **kinase,** the reagent is typically an antibody. Such kits also contain a reporter group suitable for direct or indirect detection of the reagent (i.e., the reporter group may be covalently bound to the reagent or may be bound to a second molecule, such as Protein A, Protein G, immunoglobulin or lectin, which is itself capable of binding to the reagent). Suitable reporter groups include, but are not limited to, enzymes (e.g., horseradish

peroxidase), substrates, cofactors, inhibitors, dyes, radionuclides, luminescent groups, fluorescent groups and biotin. Such reporter groups may be used to directly or indirectly detect binding of the reagent to a sample component using standard methods known to those of ordinary skill in the art.

Detail Description Paragraph - DETX:

[0088] In yet another aspect, **IKK** signalsome may be used to identify one or more native upstream **kinases** (i.e., **kinases** that phosphorylate and activate **IKK** signalsome in vivo) or other regulatory molecules that affect I.kappa.B **kinase** activity (such as phosphatases or molecules involved in ubiquitination), using methods well known to those of ordinary skill in the art. For example, **IKK** signalsome components may be used in a yeast two-hybrid system to identify proteins that interact with such components. Alternatively, an expression library may be screened for cDNAs that phosphorylate **IKK** signalsome or a component thereof.

Detail Description Paragraph - DETX:

[0090] This example illustrates the recruitment of NF.kappa.B into a protein complex (the **IKK** signalsome) containing I.kappa.B **kinase** and other signaling proteins.

Detail Description Paragraph - DETX:

[0092] As shown in FIG. 1A, I.kappa.B.alpha. in cell extracts from unstimulated cells eluted with an apparent molecular weight of .about.300 kDa (lanes 5-7), consistent with the chromatographic properties of the inactive NF.kappa.B-I.kappa.B complex (Baeuerle and Baltimore, Genes Dev. 3:1689-98, 1989). In contrast, phosphorylated I.kappa.B.alpha. (from cells stimulated for periods too short to permit complete degradation of the protein) migrated at .about.600 kDa on the same chromatography columns (lanes 2, 3). This difference in migration was specific for I.kappa.B, since analysis of the same fractions indicated that the Jun N-terminal **kinases** JNK1 and JNK2 migrated with low apparent molecular weight and showed no difference in chromatographic behavior between stimulated and unstimulated cells. Stimulation-dependent recruitment of I.kappa.B into this larger protein complex corresponded with the appearance of phosphorylated I.kappa.B, suggesting that the complex contained the specific I.kappa.B **kinases** that mediate I.kappa.B phosphorylation. These results demonstrate that that NF.kappa.B activation involves recruitment into a protein complex (the **IKK** signalsome) containing I.kappa.B **kinase** and other signaling proteins.

Detail Description Paragraph - DETX:

[0095] NF.kappa.B activation is known to occur under conditions that also stimulate MAP **kinase** pathways (Lee et al., Cell 88:213-22, 1997; Hirano, et al., J. Biol. Chem. 271:1323438, 1996). Accordingly, further experiments were

performed to detect proteins associated with MAP **kinase** and phosphatase cascades at various stages of purification of the **IKK** signalsome. In addition to RelA and I.kappa.B.beta., MEKK-1 and two tyrosine-phosphorylated proteins of .about.55 and .about.40 kDa copurified with I.kappa.B **kinase** activity (FIG. 1C). Antibodies to Rel A and I.kappa.B.beta. were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.), and antibodies to MEKK-1 were obtained from Upstate Biotechnology (Lake Placid, N.Y.). Other signaling components, including PKC.zeta., PP1 and PP2A, were detected in the same fractions as the I.kappa.B **kinase** in early chromatographic steps but did not copurify at later chromatographic steps (data not shown). Most interestingly, an unidentified protein of .about.50 kDa, detected by its crossreaction with an antibody to MKP-1, copurified with I.kappa.B **kinase** through several purification steps (FIG. 1C). This protein is unlikely to be MKP-1 itself, since the molecular weight of authentic MKP-1 is 38 kDa.

Detail Description Paragraph - DETX:

[0120] Of a large panel of antibodies tested, one of three anti-MKP-1 antibodies efficiently co-immunoprecipitated an inducible I.kappa.B **kinase** activity from HeLa cells as well as primary **human** umbilical vein endothelial cells (HUVEC). The co-immunoprecipitated **kinase (IKK** signalsome **kinase**) was inactive in unstimulated HeLa cells, but was rapidly activated within minutes of TNF.alpha. stimulation (FIG. 8A, top panel). The **IKK** signalsome **kinase** did not phosphorylate a mutant GST-I.kappa.B.alpha. protein in which serine residues 32 and 36 had been mutated to threonine (FIG. 8A top panel, even-numbered lanes). Activation of the signalsome **kinase** was maximal at 5 minutes and declined thereafter, a time course consistent with the time course of I.kappa.B.alpha. phosphorylation and degradation under the same conditions (FIG. 8A, bottom panel). As expected, the signalsome I.kappa.B **kinase** was also activated by stimulation of cells with IL-1 or PMA (FIG. 8B, lanes 1-4); moreover, its activity was inhibited in cells treated with TPCK, a known inhibitor of NF.kappa.B activation (FIG. 8B, lane 7). Additionally, the **IKK** signalsome **kinase** specifically phosphorylated full-length wild-type I.kappa.B.alpha., but not a mutant I.kappa.B.alpha. bearing the serine 32, 36 to alanine mutations, in the context of a physiological RelA-I.kappa.B.alpha. complex (FIG. 8C, lanes 3, 4). Together these results indicate that the anti-MKP-1 antibody co-immunoprecipitated the **IKK** signalsome. The signalsome-associated I.kappa.B **kinase** met all the criteria expected of the authentic I.kappa.B **kinase** and had no detectable I.kappa.B.alpha. C-terminal **kinase** activity.

Detail Description Paragraph - DETX:

[0121] The specificity of the **IKK** signalsome **kinase** was further established by kinetic analysis and by examining its activity on various peptides and recombinant protein substrates (FIG. 9A). For these studies, synthetic peptides (Alpha Diagnostics International, San Antonio, Tex.) were prepared with the following sequences:

[0124] The **kinase** displayed normal Michaelis-Menten kinetics, suggesting that it was not a mixture of diverse unrelated **kinases**. The **kinase** was capable of phosphorylating an I.kappa.B.alpha. (21-41) peptide (FIGS. 9A and 9B)) as well as two different I.kappa.B.alpha. (21-41) peptides, each bearing a free serine at either position 32 or 36 and phosphoserine at the other position (FIGS. 9A and 9B, lanes 2, 3). As expected, a peptide with phosphoserines at both positions was not phosphorylated at all (FIG. 9B, top), indicating that there was no significant turnover of the phosphates under our reaction conditions. These experiments indicated that both serines 32 and 36 were phosphoacceptor sites for the **IKK** signalsome **kinase**, thus distinguishing it from other **kinases** such as pp90Rsk which phosphorylates I.kappa.B.alpha. only at serine 32 (Schouten, et al., EMBO J. 16:3133-44, 1997).

Detail Description Paragraph - DETX:

[0125] Although the **IKK** signalsome **kinase** efficiently phosphorylated I.kappa.B peptides, it did not phosphorylate the c-Fos phosphopeptide containing a free serine and a free threonine (FIG. 9B, top), two c-Jun peptides containing serine 63 and 73, respectively, (FIG. 9A, top panel, lanes 4, 5), or an MKP-1 peptide containing four serines and three threonines (FIG. 9A, lane 6). The latter peptides were substrates for JNK2 (FIG. 9A, bottom panel, lanes 4-6). An I.kappa.B.alpha. (21-41) peptide in which serines 32 and 36 were replaced by threonines was phosphorylated by the signalsome at least 10-fold less well than the wild-type serine-containing peptide, consistent with the slower phosphorylation and degradation kinetics of I.kappa.B.alpha.(S32/36 to T) in cells (DiDonato et al., Mol. Cell. Biol. 16:1295-1304, 1996), and the preference of the **kinase** for serine over threonine at positions 32, 36 in both full-length I.kappa.B.alpha. and GST-I.kappa.B.alpha. (1-54) (FIGS. 8A and C). In addition, the **kinase** phosphorylated GST-I.kappa.B.beta. (1-54), albeit with lower affinity. In most experiments, I.kappa.B **kinase** activity was also associated with strong RelA **kinase** activity (FIG. 8C, lanes 3, 4), but no activity was observed towards several other substrates including myelin basic protein (MBP), GST-ATF2 (1-112), GST-cJun (1-79), GST-ERK3, GST-Elk (307-428), GST-p38, and a GST fusion protein containing the C-terminal region of I.kappa.B.alpha. (242-314).

Detail Description Paragraph - DETX:

[0126] The specificity of the **IKK** signalsome **kinase** was further emphasized by its susceptibility to product inhibition (FIG. 9B, bottom). The **kinase** was strongly inhibited by a doubly-phosphorylated I.kappa.B.alpha. peptide bearing phosphoserines at both positions 32 and 36, but not by the unrelated c-Fos phosphopeptide that contained a single phosphothreonine. The singly-phosphorylated and the unphosphorylated I.kappa.B.alpha. peptides were less effective inhibitors.

Detail Description Paragraph - DETX:

[0127] This example illustrates the absence of detectable free ubiquitin with a **IKK** signalsome prepared as in Example 3. Standard western blot procedures were performed (Amersham Life Science protocol, Arlington Heights, Ill.). 100 ng ubiquitin, 10 ng ubiquitin and 20 .mu.l purified IkB.alpha. **kinase** complex was subjected to 16% Tricine SDS-PAGE (Novex, San Diego, Calif.), transferred to Hybond ECL Nitrocellulose membrane (Amersham Life Science, Arlington Heights, Ill.), and probed with antibodies directed against ubiquitin (MAB1510; Chemicon, Temecula, Calif.). The results are shown in FIG. 10. Free ubiquitin could not be detected in the purified IkB.alpha. **kinase** preparation (even at very long exposures). The complexes described herein do not require addition of endogenous ubiquitin to detect IkB.alpha. **kinase** activity, nor is free ubiquitin a component in the purified IkB.alpha. **kinase** preparations of the present invention.

Detail Description Paragraph - DETX:

[0128] This Example illustrates a two-step affinity method for purification of the **IKK** signalsome, based on its recognition by the MKP-1 antibody (depicted in FIG. 11A) and the identification of I.kappa.B **kinases**.

Detail Description Paragraph - DETX:

[0129] For large scale **IKK** signalsome purification, HeLa S3 cells were stimulated for 7 minutes with 20 ng/ml TNF.alpha. (R&D Systems, Minneapolis, Minn.), harvested, whole cell lysates were prepared (1.2 g total protein) and approximately 5 mg of anti-MKP-1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.) was added and incubated at 4.degree. C. for 2 hours with gentle rotation. Subsequently, 50 ml of Protein A agarose (Calbiochem, San Diego, Calif.) was added and the mixture was incubated for an additional 2 hours. The immunoprecipitate was then sequentially washed 4.times.Pull-Down Buffer, 2.times.RIPA buffer, 2.times.Pull-Down Buffer, 1.times.3.5 M urea-Pull-Down Buffer and 3.times.Pull-Down Buffer. The immunoprecipitate was then made into a thick slurry by the addition of 10 ml of Pull-Down Buffer, 25 mg of the specific MKP-1 peptide to which the antibody was generated (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.) was added, and the mixture was incubated overnight at 4.degree. C. with gentle rotation. The eluted **IKK** signalsome was then desalted on PD 10 desalting columns (Pharmacia Biotech, Piscataway, N.J.) equilibrated with 50 mM Q buffer and chromatographed on a Mono Q column (Pharmacia Biotech, Piscataway, N.J.). Fractions containing peak IkB **kinase** activity were pooled, concentrated and subjected to preparative SDS-PAGE. The intensity of two prominent protein bands of .about.85 and .about.87 kDa (indicated by silver stain in FIG. 11B as **IKK-1 and IKK-2** respectively) correlated with the profile of I.kappa.B **kinase** activity.

Detail Description Paragraph - DETX:

[0130] Coomassie stained .about.85 and .about.87 kDa bands were excised, in-gel digested with trypsin (Wilm et al., Nature 37:466-69, 1996) and a small aliquot of the supernatant was analyzed by high mass accuracy MALDI peptide mass

mapping, as described by Shevchenko et al., Proc. Natl. Acad. Sci. USA 93:14440-45, 1996. The peptide mass map from the **IKK-1** band was searched against a comprehensive protein sequence database using the program PeptideSearch developed in house at EMBL Heidelberg. Eight measured peptide masses matched calculated tryptic peptide masses from CHUK (conserved helix-loop-helix ubiquitous **kinase**; Connely and Marcu, Cell. Mol. Biol. Res. 41:537-49, 1995) within 30 ppm, unambiguously identifying the protein. The peptide mass map of the **IKK-2** band did not result in a clear identification and therefore the sample was subjected to nanoelectrospray mass spectrometry (Wilm and Mann, Anal. Chem. 68:1-8, 1996). The peptide mixture obtained after extraction of the gel piece was micropurified on a capillary containing 50 nL of POROS R2 resin (PerSeptive Biosystems, Framingham, Mass.). After washing, the peptides were step-eluted with 0.5 .mu.l of 50% MeOH in 5% formic acid into a nanoelectrospray needle. This needle was transferred to an APIII mass spectrometer (Perkin-Elmer, Sciex, Toronto, Canada) and the sample sprayed for approximately 20 minutes. During this time, peptide ions apparent from the mass spectrum were selected and isolated in turn and fragmented in the collision chamber of the mass spectrometer. From the tandem mass spectra, short stretches of sequence were assembled into peptide sequence tags (Mann and Wilm, Anal. Chem. 66:4390-99, 1994) and searched against a protein sequence database or against dbEST using PeptideSearch.

Detail Description Paragraph - DETX:

[0131] Three peptides matched the **IKK-1** sequence. A1: IIDLGYAK (SEQ ID NO:17); A2: VEVALSNIK (SEQ ID NO:18); A3 SIQLDLER (SEQ ID NO:19). Three other peptides matched **human** EST sequences in dbEST: B1: ALELLPK (SEQ ID NO:20), B2: VIYTQLSK (SEQ ID NO:21), B6: LLLQAIQSFEK (SEQ ID NO:22) all match EST clone AA326115. The peptide B4 with the sequence LGTGGFGNVIR (SEQ ID NO:23) was found in clone R06591. After the full-length **IKK-2** sequence was obtained (as described below) two more peptides B3: ALDDILNLK (SEQ ID NO:24) and B5: DLKPENIVLQQGEQR (SEQ ID NO:25) were found in the sequence. Peptide A1 is present in both **IKK-1 and IKK-2** sequences. All the EST clones identified were clearly homologous to **human and mouse** CHUK, a serine/threonine **kinase** of hitherto unknown function. Once the complete coding sequence of **IKK-2** was obtained (as described below), all sequenced peptides (apart from two peptides derived from **IKK-1**) could be assigned to this protein.

Detail Description Paragraph - DETX:

[0132] Representative mass spectra are shown in FIGS. 12A and 12B. In FIG. 12A, peaks labeled A were matched to the tryptic peptides of **IKK-1** upon fragmentation followed by database searching with peptide sequence tags. Peaks labeled B2, B4, B6 were not found in protein databases but instead matched **human** EST sequences. One more peptide (B1) matching a **human** EST clone was observed at m/z 392.2 and is not shown in panel A. In FIG. 12B, a continuous series of C-terminal-containing fragments (Y"-ions) was used to construct a peptide sequence tag as shown by boxed letters. Search of this tag resulted in a match to the peptide LLLQALQSFEK (SEQ ID NO:22) in **human** EST clone AA326115. Two more peptides, B1 (ALELLPK; SEQ ID NO:20) and B2 (VIYTQLSK; SEQ ID NO:21)

were found in the sequence of the same EST clone.

Detail Description Paragraph - DETX:

[0133] Full-length **human IKK-1 and IKK-2** cDNAs were cloned based on the. **human** EST clones, which were obtained from Genome Systems, Inc. (St. Louis, Mo.). The precise nucleotide sequences were determined and used to design primers to PCR clone **human IKK-2 from a human** HeLa cell cDNA library (Clontech, Inc., Palo Alto, Calif.). Several **IKK-2** cDNA clones were isolated and sequenced. Full-length **mouse IKK-1** and a partial **human IKK-1** nucleotide sequence was available in the comprehensive database, primers were designed to PCR clone the respective **human and mouse IKK-1** cDNAs. The partial **human IKK-1** coding region was used to probe a HeLa cDNA phage library (Stratagene, Inc., La Jolla, Calif.) to obtain the full-length **human IKK-1** cDNA clone using standard procedures.

Detail Description Paragraph - DETX:

[0134] Sequence analysis of these clones revealed that **IKK-1 and IKK-2** were related protein serine **kinases** (51% identity) containing protein interaction motifs (FIG. 13A). Both **IKK-1 and IKK-2** contain the **kinase** domain at the N-terminus, and a leucine zipper motif and a helix-loop-helix motif in their C-terminal regions (FIG. 13A). Northern analysis indicated that mRNAs encoding **IKK-2** were widely distributed in **human** tissues, with transcript sizes of .about.4.5 kb and 6 kb (FIG. 13B). The distribution of **IKK-1** (CHUK) transcripts has been reported previously (Connely et al., Cell. Mol. Biol. Res. 41:537-49, 1995). **IKK-1 and IKK-2** mRNAs are constitutively expressed in Jurkat, HeLa and HUVEC cell lines, and their levels are not altered for up to 8 hours following stimulation with NF.kappa.B inducers such as TNF.alpha. (HeLa, HUVEC) or anti-CD28 plus PMA (Jurkat).

Detail Description Paragraph - DETX:

[0135] To further characterize the properties of **IKK-1 and IKK-2,** recombinant HA-tagged **IKK-1** and Flag-tagged **IKK-2,** either separately or alone, were in vitro transcribed and translated in wheat germ or rabbit reticulocyte lysate (Promega, Madison, Wis.). The reactions were performed exactly as described in the manufacturer's protocol. Epitope-tagged **IKK-1 and IKK-2** then immunoprecipitated with the appropriate anti-tag antibody. Immunoprecipitates containing these proteins phosphorylated I.kappa.B.alpha. and I.kappa.B.beta. with the correct substrate specificity (i.e., immunoprecipitates of **IKK-1 and IKK-2** phosphorylated both GST-I.kappa.B.alpha. (FIG. 14A, panel 3) and GST-I.kappa.B.beta. (panel 4), but did not phosphorylate the corresponding S32/36 to T mutant protein). **IKK-1** expressed in rabbit reticulocyte lysates was also capable of autophosphorylation (FIG. 14A, panel 2, lane 1), whereas a **kinase**-inactive version of **IKK-1,** in which the conserved lysine 44 had been mutated to methionine, showed no autophosphorylation. In contrast **IKK-2,** although expressed at equivalent levels in the lysates (panel 1), showed very weak autophosphorylation (panel 2, lane 2).

Detail Description Paragraph - DETX:

[0136] Expression of the **kinase** inactive mutants (K to M) of **IKK-1 and IKK-2** indicate that both play critical roles in NF.kappa.B activation as demonstrated by immunofluorescent studies (FIGS. 14B and 14C). For these studies, HeLa cells were transiently transfected with either HA-tagged **IKK-1** or Flag-tagged **IKK-2**. Cells were fixed for 30 minutes with methanol. For immunofluorescence staining, the cells were incubated sequentially with primary antibody in PBS containing 10% donkey serum and 0.25% Triton X-100 for 2 hours followed by fluorescein-conjugated or Texas red-conjugated secondary antibody (Jackson Immunoresearch Laboratories, Inc., West Grove, Pa.; used at 1:500 dilution) for 1 hour at room temperature. The coverslips were rinsed and coverslipped with Vectashield (Vector Laboratories, Burlingame, Calif.) before scoring and photographing representative fields. Primary antibodies used for immunofluorescence staining included antibodies against Rel A (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.), HA tag (Babco, Berkeley, Calif.) and Flag tag (IBI-Kodak, New Haven, Conn.).

Detail Description Paragraph - DETX:

[0137] **Kinase**-inactive versions (K44 to M) of **IKK-1 and IKK-2** had no effect on the subcellular localization of RelA in unstimulated HeLa cells, since RelA remained cytoplasmic both in cells expressing the epitope-tagged proteins and in the adjacent untransfected cells (FIGS. 14B and 14C, top panels). In contrast, both mutant proteins inhibited RelA nuclear translocation in TNF.alpha.-stimulated cells (FIGS. 14B and 14C, bottom panels). The inhibition mediated by the **IKK-2** mutant was striking and complete (FIG. 14C: compare mutant **IKK**-2-expressing cells with untransfected cells in the same field), whereas that mediated by the mutant **IKK-1** protein, expressed at apparently equivalent levels, was significant but incomplete (FIG. 14B). This difference in the functional activities of the two mutant **kinases** may point to distinct roles for these two **kinases** in NF.kappa.B activation.

Detail Description Paragraph - DETX:

[0139] Both **IKK-1 and IKK-2 kinases** were active when expressed in wheat germ extracts, since they were capable of autophosphorylation, but they were inactive with respect to phosphorylation of I.kappa.B substrates. Since both autophosphorylation and substrate phosphorylation were intact in rabbit reticulocyte lysates, there appeared to be a direct correlation between the association of **IKK-1 and IKK-2** into a higher order protein complex and the presence of specific I.kappa.B **kinase** activity in **IKK-1 and IKK-2** immunoprecipitates. This higher order complex is most likely the **IKK** signalsome itself. Indeed, immunoprecipitation of rabbit reticulocyte lysates with anti-MKP-1 antibody pulls down a low level of active I.kappa.B **kinase** activity characteristic of the **IKK** signalsome.

Detail Description Paragraph - DETX:

[0140] It is clear that the **IKK** signalsome contains multiple protein components in addition to **IKK-1 and IKK-2** (FIG. 11B). Some of these may be upstream **kinases** such as MEKK-1 (Chen et al., Cell 84:853-62, 1996) or NIK (Malinin, et al., Nature 385:540-44, 1997); others may be adapter proteins that mediate the **IKK**-1:IKK-2 interaction. Indeed MEKK-1 copurifies with **IKK** signalsome activity (FIG. 1C), and two other signalsome proteins have been functionally identified. The protein crossreactive with anti-MKP-1 is an intrinsic component of the **IKK** signalsome **kinases,** since the I.kappa.B **kinase** activity coprecipitated with this antibody is stable to washes with 2-4 M urea Moreover, both **IKK-1** immunoprecipitates and MKP-1 immunoprecipitates containing the **IKK** signalsome (FIG. 8C) contain an inducible RelA **kinase** whose kinetics of activation parallel those of the I.kappa.B **kinase** in the same immunoprecipitates. Another strong candidate for a protein in the signalsome complex is the E3 ubiquitin ligase that transfers multiubiquitin chains to phosphorylated I.kappa.B (Hershko et al., Annu. Rev. Biochem. 61:761-807,1992).

Detail Description Paragraph - DETX:

[0141] These results indicate that **IKK-1 and IKK-2** are functional **kinases** within the **IKK** signalsome, which mediate I.kappa.B phosphorylation and NF.kappa.B activation. Appropriate regulation of **IKK-1 and IKK-2** may require their assembly into a higher order protein complex, which may be a heterodimer facilitated by adapter proteins, the complete **IKK** signalsome, or some intermediate subcomplex that contains both **IKK-1 and IKK-2**.

Claims Text - CLTX:

2. An **IKK** signalsome according to claim 1 wherein the signalsome is derived from a **human** tissue or cell line.

Claims Text - CLTX:

10. A method for phosphorylating a substrate of an **IKK** signalsome, comprising contacting a substrate with a polypeptide comprising a component of an **IKK** signalsome having I.kappa.B **kinase** activity, and thereby phosphorylating the substrate.

Claims Text - CLTX:

15. A method according to claim 14, wherein the **IKK** signalsome activity modulated is selected from the group consisting of I.kappa.B **kinase** activity, p65 **kinase** activity and **IKK** phosphatase activity.

Claims Text - CLTX:

23. The method of claim 21, wherein the agent inhibits **kinase** activity of an activated **IKK** signalsome.

Claims Text - CLTX:

30. A method for identifying an upstream **kinase** in the NF-.kappa.B signal transduction cascade, comprising evaluating the ability of a candidate upstream **kinase** to phosphorylate and induce enzymatic activity of an **IKK** signalsome or a component or variant thereof, and thereby identifying an upstream **kinase** in the NF-.kappa.B signal transduction cascade.

Claims Text - CLTX:

32. A method for preparing an **IKK** signalsome from a biological sample, comprising: (a) separating a biological sample into two or more fractions; and (b) monitoring I.kappa.B **kinase** activity in the fractions.

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PGPUB-FILING-TYPE: new

DOCUMENT-IDENTIFIER: US 20020150947 A1

TITLE: Extended tethering approach for rapid identification of ligands

PUBLICATION-DATE: October 17, 2002

INVENTOR-INFORMATION:

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APPL-NO: 09/ 990421

DATE FILED: November 21, 2001

RELATED-US-APPL-DATA:

non-provisional-of-provisional 60252294 20001121 US
non-provisional-of-provisional 60310725 20010807 US

US-CL-CURRENT: 435/7.1,435/6 ,436/518

ABSTRACT:

The invention concerns a method for rapid identification and characterization of binding partners for a target molecule, and for providing binding partners with improved binding affinity. More specifically, the invention concerns an improved tethering method for the rapid identification of at least two binding partners that bind near one another to a target molecule.

[0001] This application is a continuation-in-part and claims priority under 35 U.S.C. .sctn. 1.19(e) of U.S. Provisional Application No. 60/252,294 filed on Nov. 21, 2000 and U.S. Provisional Application No. 60/310,725 filed on Aug. 7, 2001.

---------- KWIC ---------

Detail Description Paragraph - DETX:

[0074] An important group of human inflammation and immunology targets includes: IgE/IgER, ZAP-70, lck, syk, ITK/BTK, TACE, Cathepsin S and F, CD11a, LFA/ICAM, VLA-4, CD28/B7, CTLA4, TNF alpha and beta, (and the p55 and p75 TNF

receptors), CD40L, p38 map **kinase,** IL-2, IL-4, 11-13, IL-15, Rac 2, PKC theta, IL-8, TAK-1, jnk, **IKK2** and IL-18.

Detail Description Paragraph - DETX:

[0075] Still other important specific targets include: caspases 1, 3, 8 and 9, IL-1/IL-1 receptor, BACE, HIV integrase, PDE IV, Hepatitis C helicase, Hepatitis C protease, rhinovirus protease, tryptase, cPLA (cytosolic Phospholipase A2), CDK4, c-jun **kinase,** adaptors such as Grb2, GSK-3, AKT, MEKK-1, PAK-1, raf, TRAF's 1-6, Tie2, ErbB 1 and 2, FGF, PDGF, PARP, CD2, C5a receptor, CD4, CD26, CD3, TGF-alpha, NF-KB, **IKK** beta, STAT 6, Neurokinnin-1, PTP-1B, CD45, Cdc25A, SHIP-2, TC-PTP, PTP-alpha, LAR and **human** p53, bax/bc12 and mdm2.

Claims Text - CLTX:

8. The process of claim 6 wherein said protein is selected from the group consisting of IgE/IgER, ZAP-70, lck, syk, ITK/BTK, TACE, Cathepsin S and F, CD11a, LFA/ICAM, VLA-4, CD28/B7, CTLA4, TNF alpha and beta, (and the p55 and p75 TNF receptors), CD40L, p38 map **kinase,** IL-2, IL-4, 11-13, IL-15, Rac 2, PKC theta, IL-8, TAK-1, jnk, **IKK2,** IL-18, caspases 1, 3, 8 and 9, IL-1/IL-1 receptor, BACE, HIV integrase, PDE IV, Hepatitis C helicase, Hepatitis C protease, rhinovirus protease, tryptase, cPLA (cytosolic Phospholipase A2), CDK4, c-jun **kinase,** adaptors such as Grb2, GSK-3, AKT, MEKK-1, PAK-1, raf, TRAF's 1-6, Tie2, ErbB 1 and 2, FGF, PDGF, PARP, CD2, C5a receptor, CD4, CD26, CD3, TGF-alpha, NF-kB, **IKK** beta, STAT 6, Neurokinin-1, PTP-1B, CD45, Cdc25A, SHIP-2, TC-PTP, PTP-alpha, LAR and **human** p53, bax/bcl2 and mdm2.

PGPUB-DOCUMENT-NUMBER: 20020127673

PGPUB-FILING-TYPE: new

DOCUMENT-IDENTIFIER: US 20020127673 A1

TITLE: Nod2 nucleic acids and proteins

PUBLICATION-DATE: September 12, 2002

INVENTOR-INFORMATION:

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APPL-NO: 10/ 014269

DATE FILED: October 26, 2001

RELATED-US-APPL-DATA:

non-provisional-of-provisional 60244289 20001030 US

US-CL-CURRENT: 435/183,435/320.1 ,435/325 ,435/410 ,435/69.1 ,536/23.2 ,800/278 ,800/8

ABSTRACT:

The present invention relates to intracellular signalling molecules, in particular the Nod2 protein and nucleic acids encoding the Nod2 protein. The present invention provides isolated nucleotide sequence encoding Nod2, isolated Nod2 peptides, antibodies that specifically bind Nod2, methods for the detection of Nod2, and methods for screening compounds for the ability to alter Nod2 associated signal transduction.

[0001] This application claims priority to U.S. provisional patent application serial No. 60/244,289, which is herein incorporated by reference in its entirety. This patent application was supported in part by grant CA-64556 from the National Institutes of Health. The government has certain rights in the invention.

---------- KWIC ---------

Detail Description Paragraph - DETX:

[0352] In the experimental disclosure which follows, the following abbreviations apply: eq (equivalents); M (Molar); .mu.M (micromolar); N

(Normal); mol (moles); mmol (millimoles); .mu.mol (micromoles); nmol (nanomoles); g (grams); mg (milligrams); .mu.g (micrograms); ng (nanograms); l or L (liters); ml (milliliters); .mu.l (microliters); cm (centimeters); mm (millimeters); .mu.m (micrometers); nm (nanometers); .degree. C. (degrees Centigrade); U (units), mU (milliunits); min. (minutes); sec. (seconds); % (percent); kb (kilobase); bp (base pair); PCR (polymerase chain reaction); BSA (bovine serum albumin); Fisher (Fisher Scientific, Pittsburgh, Pa.); Sigma (Sigma Chemical Co., St. Louis, Mo.); Promega (Promega Corp., Madison, Wis.); Perkin-Elmer (Perkin-Elmer/Applied Biosystems, Foster City, Calif.); Boehringer Mannheim (Boehringer Mannheim, Corp., Indianapolis, Ind.); Clonetech (Clonetech, Palo Alto, Calif.); Qiagen (Qiagen, Santa Clarita, Calif.); Stratagene (Stratagene Inc., La Jolla, Calif.); National Biosciences (National Biosciences Inc, Plymouth Minn.)and NEB (New England Biolabs, Beverly, Mass.), CARD (caspase-recruitment domain); EST (expressed sequence tag); HA (hemagglutinin); I.kappa.B (inhibitor of NF-.kappa.B); **IKK** (I.kappa.B **kinase**); LRRs (leucine-rich repeats); NBD (nucleotide-binding domain); NF-.kappa.B (nuclear factor .kappa.B); TNF.alpha. (tumor necrosis factor .alpha.); wt (wild-type); Ab (antibody); IL-1 (interleukin 1); IL-1R (IL-1 receptor); LPS (lipopolysaccharide); LTA (lipoteichoic acid); PGN (peptidoglycan); SBLP (synthetic bacterial lipoprotein); and TLR (Toll-like receptor).

Detail Description Paragraph - DETX:

[0361] The Nod2 cDNA was cloned into pcDNA3-HA and pcDNA3-Fpk3-Myc (Inohara et al., [2000], supra). Deletion and sitedirected mutants of Nod2 (129-1040, A125-214, 1-125, 1-301, 1-744, 265-1040, 126-301, 265744, 744-1040, K305R, 1-744K305R) were constructed by a PCR method and cloned into pcDNA3-HA and pcDNA3-Fpk3-Myc (Inohara et al., [2000], supra). The authenticity of all constructs was confirmed by sequencing. pcDNA3-Flag-RICK, pcDNA3-Flag-RICK(1-374), pcDNA3-Flag-RICK(374-540), pcDNA3-Myc-RICK(406-540), pcDNA3-Myc-RIP(558-671), pRK7-Flag-IKK.alpha., pRK7-FlagIKK.alpha.-K44A, RSVMad-3MSS(I.kappa.-B.alpha.-S32A/S36A), pRK7-Flag-IKK.beta., pRK7-Flag-IKK.beta.-K44A, and pcDNA3-Flag-IKK.gamma.(134-419) have been described previously (Inohara et al., supra, 10). The expression plasmids pcDNA3-Nod1-Flag, pcDNA3-Nod1 (1-648)-Flag, pcDNA3-Flag-**IKKi**, pcDNA3CIPER-Flag, pCMV-ILIR, pCMV-TLR4-Flag, pcDNA3-Flag-RIP, pcDNA3-MyD88 DN(amino acids 1-109), pcDNA3-CD14, pCMV-MD2-FLAG and pcDNA3-p-gal have also been described previously (Inohara et al., [1999], Supra; Inohara et al., [1999], Supra; Inohara et al., [2000], supra; Shimada et al., Int. Immunol., 11:1357-1362 [1999]; Huang et al., PNAS, 94:12829-12832 [1997]; Medzhitov et al., Mol. Cell, 2:253-258 [1998]; Hsu et al., Immunity, 4:387-396 [1996]). To construct the expression plasmid producing C-terminally HA-tagged mature interleukin-1 P (IL1.beta.), pcDNA3-mIL1.beta.-HA, the mature region of **mouse** IL1.beta. was amplified by PCR and inserted into pcDNA3-HA-pro which contains the signal sequence of protrypsin and the HA tag.

Detail Description Paragraph - DETX:

[0373] This example demonstrates that NF-.kappa.B activation induced by Nod2 requires IKK.gamma. and is inhibited by dominant negative forms of **IKKs** and RICK. A main pathway of NF-.kappa.B activation is mediated by I.kappa.B

kinases (IKKS) resulting in I.kappa.B phosporylation and release of cytoplasmic NF-.kappa.B (Karin, J. Biol. Chem. 274: 27339-27342 [1999]). To determine whether Nod2 activates an **IKK**-dependent pathway, Nod2 was co-expressed with mutant forms of IKK.alpha., IKK.beta., and I.kappa.B that have been shown to act as dominant inhibitors of their corresponding endogenous counterparts and/or the **IKK** complex (Karin, supra). In addition, a truncated mutant of IKK.gamma./Nemo (residues 134-419) was used that is defective in IKK.alpha. and IKK.beta. binding and acts as an inhibitor of NF-.kappa.B activation induced by RIP and RICK (Inohara et al., [2000], supra). The NF-.kappa.B activity induced by Nod2 as well as that induced by TNF.alpha. stimulation were greatly inhibited by mutant IKK.alpha., IKK.beta., IKK.gamma., and I.kappa.B.alpha. (FIG. 5A). Because RICK has been shown to serve as a downstream target of Nod1 (Bertin et al., supra, Inohara et al., [1999] supra, Inohara et al., [2000], supra), a truncated form of RICK containing its CARD (residues 406-540) that acts as a dominant inhibitor of Nod1 activity (Bertin et al., supra) was used to test whether NF-.kappa.B activation induced by Nod2 is similarly inhibited by this RICK mutant. NF-.kappa.B activation induced by Nod2 was inhibited by mutant RICK but not by a mutant form of RIP that expresses its death effector domain (FIG. 5A). The inhibition by the CARD of RICK was specific in that it did not interfere with ability of TNF.alpha. to induce NF-.kappa.B, an activity that was inhibited by the RIP mutant (FIG. 5A). To verify that Nod2 acts upstream of the **IKK** complex to activate NF-.kappa.B, we tested the ability of Nod2 to activate NF-.kappa.B in parental Rat1 fibroblasts and 5R cells, a Rat1 derivative cell line that is defective in IKK.gamma., an essential subunit of the **IKKs** (Yamaoka et al., supra). Nod2, as well as Nod1 and TNFoc, induced NF-KB activity in parental Rat1 cells but not in IKK.gamma.-deficient 5R cells (FIG. 5B). As a control, expression of IKK.beta., which functions downstream of IKK.gamma., induced NF-.kappa.B activation in both Rat1 and 5R cell lines (FIG. 5B). These results indicate that Nod2 acts through IKK.gamma./IKK/IKK.beta. to activate NF-.kappa.B.

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PGPUB-FILING-TYPE: new

DOCUMENT-IDENTIFIER: US 20020127654 A1

TITLE: Compositions and methods for production cell culture

PUBLICATION-DATE: September 12, 2002

INVENTOR-INFORMATION:

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APPL-NO: 10/ 080428

DATE FILED: February 22, 2002

RELATED-US-APPL-DATA:

non-provisional-of-provisional 60270943 20010222 US

US-CL-CURRENT: 435/69.5,435/320.1 ,435/325

ABSTRACT:

The invention provides improved methods of recombinant protein production in cell culture. More specifically, the invention relates to the activation of NF-kappa-B transcription factor complex in cells so as to improve production characteristics.

RELATED APPLICATION DATA

[0001] This application claims the benefit of provisional U.S. application No. 60/270,943, filed Feb. 22, 2001, the entire disclosure of which is incorporated by reference herein.

---------- KWIC ---------

Summary of Invention Paragraph - BSTX:

[0005] Two closely related **kinases** responsible for IKB phosphorylation (and hence NF-kappa-B activation) have been identified and are termed Inhibitor-Kappa B **Kinase**-alpha (**IKK-1,** for I-Kappa-B **Kinase** 1) and Inhibitor-Kappa B **Kinase**-beta (**IKK-2,** for I-Kappa-B **Kinase** 2) (DiDonato et al., Nature, 1997, 388, 548-554; Zandi et al., Science, 1998, 281, 1360-1363; Zandi et al., Cell, 1997, 91, 243-252). In these studies, Inhibitor-Kappa B

Kinase-alpha and Inhibitor-Kappa B **Kinase**-beta were found to directly phosphorylate IKB-alpha and IKB-beta.

Summary of Invention Paragraph - BSTX:

[0021] Preferably, the cells are genetically engineered to express a NF-kappa-B gene product or an **IKK** that is homologous to, or derived from the same species, as that of the cell. However, as NF-kappa-B genes tend to be well conserved, it is expected that even expression of heterologous gene products will be advantageous. For example, in the non-limiting embodiments described below, the **human** p65 subunit was expressed in simian and **murine** cells, and successfully increased expression levels of a gene of interest.

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PGPUB-FILING-TYPE: new

DOCUMENT-IDENTIFIER: US 20020107252 A1

TITLE: Novel Compounds

PUBLICATION-DATE: August 8, 2002

INVENTOR-INFORMATION:

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APPL-NO: 09/ 868884

DATE FILED: February 5, 2002

FOREIGN-APPL-PRIORITY-DATA:

COUNTRY	APPL-NO	DOC-ID	APPL-DATE
GB	0003154.2	2000GB-0003154.2	February 12, 2000

PCT-DATA:
APPL-NO: PCT/SE01/00248
DATE-FILED: Feb 7, 2001
PUB-NO:
PUB-DATE:
371-DATE:
102(E)-DATE:

US-CL-CURRENT: 514/252.01,514/252.05 ,514/255.05 ,514/256 ,514/340 ,514/342 ,514/343 ,514/370 ,514/377 ,514/397 ,514/398 ,514/422 ,514/426 ,514/447 ,514/471 ,544/238 ,544/403 ,544/405 ,548/190 ,548/233 ,548/326.5 ,548/557

ABSTRACT:

The invention relates to heteroaromatic carboxamides of formula (I), 1

wherein A, R.sup.1, R.sup.2 and X are as defined in the specification, processes and intermediates used in their preparation, pharmaceutical compositions containing them and their use in therapy.

---------- KWIC ---------

Summary of Invention Paragraph - BSTX:

[0005] Recent publications have partially elucidated the NF-.kappa.B activation. This work has identified three key enzymes which regulate specific I.kappa.B/NF-.kappa.B interactions: NF-.kappa.B inducing **kinase** (NIK) (Boldin et. al., Cell 85:803-815, 1996), I.kappa.B **kinase**-1 (**IKK-1**) (Didonato et. al., Nature 388:548, 1997; Regnier at. al., Cell 90:373 1997) and I.kappa.B **kinase**-2 (**IKK-2**) (Woronicz et. al., Science 278:866. 1997; Zandi et. al., Cell 91:243, 1997).

Summary of Invention Paragraph - BSTX:

[0008] NIK, **IKK-1 and IKK-2** are all serine threonine **kinases**. Recent data has shown that tyrosine **kinases** also play a role in regulating the activation of NF-.kappa.B. A number of groups have shown that TNF-.alpha. induced NF-.kappa.B activation can be regulated by protein tyrosine phosphatases (PTPs) and tyrosine **kinases** (Amer et. al., JBC 273:29417-29423, 1998: Hu et. al., JBC 273:33561-33565. 1998: Kaekawa et. al., Biochem. J. 337:179-184, 1999: Singh et. al., JBC 271 31049-31054, 1996). The mechanism of action of these enzymes appears to be in regulating the phosphorylation status of I.kappa.B. For example, PTP1B and an unidentified tyrosine **kinase** appear to directly control the phosphorylation of a lysine residue (K42) on I.kappa.B-.alpha., which in turn has a critical influence on the accessibility of the adjacent serine residues as targets for phosphorylation by **IKK**.

Summary of Invention Paragraph - BSTX:

[0009] Several groups have shown that **IKK-1 and IKK-2** form part of a `signalosome` structure in association with additional proteins including IKAP (Cohen et. al., Nature 395:292-296, 1998; Rothwarf et. al., Nature 395:297-300, 1998), MEKK-1, putative MAP **kinase** phosphatase (Lee et. al., Proc. Natl. Acad. Sci. USA 95:9319-9324, 1998), as well as NIK and I.kappa.B. Data is now emerging to suggest that although both **IKK-1 and IKK-2** associate with NIK, they are differentially activated, and therefore might represent an important integration point for the spectrum of signals that activate NF-.kappa.B. Importantly, MEKK-1 (one of the components of the putative signalosome and a target for UV light, LPS induced signalling molecules and small GTPases) has been found to activate **IKK-2** but not **IKK-1**. Similarly, NIK phosphorylation of **IKK-1** results in a dramatic increase in **IKK-1** activity but only a small effect on **IKK-2** (for review, see Mercurio, F., and Manning, A. M., Current Opinion in Cell Biology, 11:226-232, 1999).

Summary of Invention Paragraph - BSTX:

[0161] The compounds of formula (I) have activity as pharmaceuticals, in particular as **IKK2** enzyme inhibitors, and may be used in the treatment (therapeutic or prophylactic) of conditions/diseases in human and non-human animals in which inhibition of **IKK2** is beneficial. Examples of such

conditions/diseases include inflammatory diseases or diseases with an inflammatory component. Particular diseases include inflammatory arthritides including rheumatoid arthritis, osteoarthritis, spondylitis, Reiters syndrome. psoriatic arthritis, lupus and bone resorptive disease: multiple sclerosis. inflammatory bowel disease including Crohn's disease; asthma, chronic obstructive pulmonary disease, emphysema, rhinitis, myasthenia gravis, Graves' disease, allograft rejection, psoriasis, dermatitis, allergic disorders, immune complex diseases, cachexia, ARDS, toxic shock, cardiovascular disorders, heart failure, myocardial infarcts, atherosclerosis, reperfusion injury, AIDS and cancer.

Detail Description Paragraph - DETX:

IKK2 Filter Kinase Assay

Detail Description Paragraph - DETX:

[0650] Compounds were tested for inhibition of **IKK2** using a filter **kinase** assay. The test compounds were dissolved to 10 mM in dimethylsulphoxide (DMSO). The compounds were then diluted 1 in 40 in **kinase** buffer (50 mM Tris, pH 7.4 containing 0.1 mM EGTA, 0.1 mM sodium orthovanadate and 0.1% .beta.-mercaptoethanol). 1 in 3 serial dilutions were made from this solution with 2.5% DMSO in **kinase** buffer. 20 .mu.l of compound dilution was added to wells of a 96 well plate in duplicate. 20 .mu.l 2.5% DMSO in **kinase** buffer instead of compound was added to control wells (0% inhibition). 20 .mu.l 0.5 M EDTA was added instead of compound to background wells (100% inhibition).

Detail Description Paragraph - DETX:

IKK1 Filter Kinase Assay

Detail Description Paragraph - DETX:

[0653] The selectivity of compounds was assessed by testing them for inhibition of **IKK1** using a filter **kinase** assay. The assay conditions were identical to the **IKK2** filter **kinase** assay except that a mixture of **IKK1** (0.25 .mu.g/well) and 1-53 GST I.kappa.B (9 .mu.g/well) was added to each well to start the reaction.

US-PAT-NO: 6518021

DOCUMENT-IDENTIFIER: US 6518021 B1

TITLE: Method for extracting quantitative information relating to an influence on a cellular response

DATE-ISSUED: February 11, 2003

INVENTOR-INFORMATION:

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Almholt; Kasper	Virum	N/A	N/A	DK
Scudder; Kurt				

APPL-NO: 09/ 417197

DATE FILED: October 7, 1999

PARENT-CASE:

RELATED APPLICATIONS This application is a continuation of International Application No. PCT/DK98/00145, filed Apr. 7, 1998, which in turn claims priority to Denmark Application No. 0392/97, filed Apr. 7, 1997 both of which are incorporated herein by reference.

FOREIGN-APPL-PRIORITY-DATA:

COUNTRY	APPL-NO	APPL-DATE
DK	0392/97	April 7, 1997

US-CL-CURRENT: 435/6; 435/320.1 ; 435/325 ; 435/354 ; 435/357 ; 435/358 ; 435/365 ; 435/366 ; 435/367 ; 536/23.1 ; 536/23.5 ; 800/13

ABSTRACT:

Cells are genetically modified to express a luminophore, e.g., a modified (F64L, S65T, Y66H) Green Flourescent Protein (GFP, EGFP) coupled to a component of an intracellular signalling pathway such as a transcription factor, a cGMP- or cAMP-dependent protein kinase, a cyclin-, calmodulin- or phospholipid-dependent or mitogen-activated serine/threonin protein kinase, a tryosine protein kinase, or a protein phosphatase (e.g. PKA, PKC, Erk, Smad, VASP, actin, p38, Jnkl, PKG, IkappaB, CDK2, Grk5, Zap70, p85, protein-tyrosine phosphatase 1C, Stat5, NFAT, NFkappaB, RhoA, PKB). An influence modulates the intracellular signaling pathway in such a way that the luminophore is being redistributed or translocated with the component in living cells in a manner experimentally determined to be correlated to the degree of influence. Measurement of redistribution is performed by recording of light intensity,

flourescence lifetime, polarization, wavelength shift, resonance energy transfer, or other properties by an apparatus consisting of e.g. a flourescence microscope and a CCD camera. Data stored as digital images are processed to numbers representing the degree of redistribution. The method can be used as a screening program for identifying a compound that modulates a component and is capable of treating a disease related to the function of the component.

88 Claims, 12 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 12

---------- KWIC ---------

Detailed Description Text - DETX:

a) The alpha subunit of the **human** IkappaB **kinase** gene (GenBank Accession number: AF009225) is amplified using PCR according to standard protocols with primers **IKK**-top (SEQ ID NO:96) and **IKK**-bottom/+stop (SEQ ID NO:98). The PCR product is digested with restriction enzymes EcoR1 and Acc65I, and ligated into pEGFP-C1 (Clontech, Palo Alto; GenBank Accession number U55763) digested with EcoR1 and Acc65I. This produces an EGFP-IkappaB-**kinase** fusion (SEQ ID NO:120 &121) under the control of a CMV promoter.

Detailed Description Text - DETX:

b) The alpha subunit of the **human** IkappaB **kinase** gene (GenBank Accession number: AF009225) is amplified using PCR according to standard protocols with primers **IKK**-top (SEQ ID NO:96) and **IKK**-bottom/-stop (SEQ ID NO:97). The PCR product is digested with restriction enzymes EcoR1 and Acc65I, and ligated into pEGFP-N1 (Clontech, Palo Alto; GenBank Accession number U55762) digested with EcoR1 and Acc65I. This produces an IkappaB-**kinase**-EGFP fusion (SEQ ID NO:122 &123) under the control of a CMV promoter.

US-PAT-NO: 6489151

DOCUMENT-IDENTIFIER: US 6489151 B1

TITLE: Biologically active alternative form of the IKK.alpha. I.kappa.B kinase

DATE-ISSUED: December 3, 2002

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Marcu; Kenneth B.	Stony Brook	NY	N/A	N/A
Connelly; Margery A.	Medford	NY	N/A	N/A

APPL-NO: 09/ 536882

DATE FILED: March 27, 2000

US-CL-CURRENT: 435/194; 536/23.2

ABSTRACT:

The present invention provides isolated I.kappa.B kinases that regulate NF.kappa.B gene transcription that lack both a leucine zipper like .alpha.-helix domain and helix-loop-helix domain. Also provided are the amino acid sequences of these kinases and the nucleotide sequence encoding these kinases, and other related protein and nucleic acid molecules.

16 Claims, 12 Drawing figures

Exemplary Claim Number: 1

Number of Drawing Sheets: 12

---------- KWIC ---------

Brief Summary Text - BSTX:

Of critical importance for elucidating, and controlling, the signaling pathways that lead to NF-.kappa.B activation is the determination and characterization of **kinases** that directly phosphorylate I.kappa.B. The abbreviation "**IKK**" is used to designate an I.kappa.B **kinase**. Recently, an I.kappa.B **kinase (IKK)**, designated IKK.alpha., was identified in a yeast-two-hybrid screen with NIK as bait. Regnier et al., Cell 90, 373-383 (1997). IKK.alpha. was also purified using conventional biochemical techniques and determined to be the major I.kappa.B **kinase** activity induced by TNF stimulation of HeLa cells. DiDonato et al., Nature 388, 548-554 (1997). IKK.alpha. had been cloned previously in a reverse transcriptase polymerase chain reaction (RT-PCR) based search for myc-like genes containing helix-loop-helix domains and was termed CHUK

(conserved helix-loop-helix ubiquitous **kinase**). Connelly and Marcu, Cellular and Molecular Biology Research 41, 537-549 (1995). CHUK was renamed IKK.alpha. when its function was discovered. Regnier et al. (1997). The identification of IKK.alpha. (CHUK) as a cytoplasmic **kinase** which phosphorylates I.kappa.B family members at their physiologically relevant sites and targets them for proteosome-mediated degradation was a major breakthrough.

Brief Summary Text - BSTX:

The IKK.alpha. (CHUK) gene encodes a 745 amino-acid polypeptide (having a molecular mass of approximately 85 kDa). **Murine and human** IKK.alpha. (CHUK) cDNA clones were found to be almost identical. Connelly and Marcu, Cellular and Molecular Biology Research 41, 537-549 (1995). Another **kinase,** termed IKK.beta., homologous to IKK.alpha., has also been reported. Stancovski and Baltimore, Cell 91, 299-302 (1997); Woronicz et al., Science 278, 866-869 (1997); and Zandi et al. Cell 91, 243-252 (1997). IKK.alpha. and IKK.beta. have 52% overall similarity to each other and 65% identity in the **kinase** domain. Zandi et al., Cell 91, 243-252 (1997). IKK.alpha. and IKK.beta. share two carboxy-proximal structural domains, leucine zipper and H-L-H. (Connelly and Marcu, 1995). Since these domains are thought to play roles in protein-protein interactions, the **IKKs** may employ these domains to recruit proteins involved in their regulation or to facilitate binding to specific substrates. Recent experiments on the regulation of IKK.beta. activation suggest that the probable interaction of the carboxy-proximal H-L-H and amino-proximal catalytic domains are required for its cytokine induced activation. (Delhase et al., 1999). An I.kappa.B **kinase** termed T2K has been described in U.S. Pat. No. 5,776,717 to Cao.

Brief Summary Text - BSTX:

IKK.alpha. (CHUK) and IKK.beta. have structural motifs characteristic of the **IKKs**. This 30 includes an amino terminal serine-threonine **kinase** domain separated from a carboxyl proximal helix-loop-helix (H-L-H) domain by a leucine zipper-like amphipathic .alpha.-helix structure. These structural characteristics are unlike other **kinases** and the domains are thought to be involved in protein-protein interactions. The **IKKs** may employ these domains to recruit proteins involved in their regulation or to facilitate binding to specific substrates. Recent experiments on the regulation of IKK.beta. activation suggest that the probable interaction of the H-L-H and the **kinase** domains are required for its cytokine-induced activation (Delhase et al., 1999).

Detailed Description Text - DETX:

Akin to IKK.alpha./CHUK, the IKK.alpha.-.DELTA.LH and IKK.alpha.-.DELTA.Cm proteins are TNF-.alpha. inducible, NF-.kappa.B activating I.kappa.B.alpha. **kinases**. By a combination of NF-.kappa.B element driven luciferase gene reporter assays, immune complex **kinase** assays and co-immunoprecipitations with other known components of the approximately 700-900 kD **IKK** complex, the

IKK.alpha.-.DELTA.LH and IKK.alpha.-.DELTA.Cm proteins were found to behave in a similar fashion to full length IKK.alpha./CHUK by several criteria. First, expression plasmid dose response curves reveal that each form of IKK.alpha./CHUK activates a comparable level of NF-.kappa.B luciferase activity even at their limiting dosages (FIG. 4B). Second, each form of IKK.alpha./CHUK correctly phosphorylates I.kappa.B.alpha. (on serines 32 and 36) in response to TNF.alpha. signaling (FIG. 5A). Third, IKK.alpha.-.DELTA.Cm activates NF-.kappa.B and phosphorylates I.kappa.B.alpha. with an enzymatic time course superimposable with full length IKK.alpha./CHUK. (FIG. 5B.) Fourth, like IKK.alpha./CHUK, IKK.alpha.-.DELTA.Cm's ability to activate NF-.kappa.B is not appreciably enhanced by co-expression with IKK.beta. and is inhibited by a **kinase** inactive, ATP binding domain mutant of IKK.alpha./CHUK. Therefore, these isoforms of IKK.alpha./CHUK, which lack the LZ and H-L-H domains, retain a number of functions of the full length IKK.alpha./CHUK. It is surprising that the carboxy-tail domain of the full length IKK.alpha./CHUK does not significantly contribute to the **kinase's** functional activity.

Detailed Description Text - DETX:

AKAP, A-**kinase** anchoring protein; CHUK, conserved helix-loop-helix ubiquitous **kinase**; GST, glutathione S-transferase; HA, hemagglutinin; **IKK**, I B **kinase**; IL, Interleukin; MEKK-1, mitogen-activated protein kinase/ERK **kinase kinase**-1; NIK, NF-KB-inducing **kinase**; RT, reverse transcriptase; TNF, tumor necrosis factor: TRAF, tumor necrosis factor receptor-associated factor.

Other Reference Publication - OREF:

Zandi et al., "The I.kappa.B **Kinase** Complex (**IKK**) Contains Two **Kinase** Subunits, IKK.alpha. and IKK.beta., Necessary for I.kappa.B Phosphorylation and NF-.kappa.B Activation", Cell vol. 91: 243-252 (1997).

US-PAT-NO: 6479266

DOCUMENT-IDENTIFIER: US 6479266 B1

TITLE: IKK-.alpha. proteins nucleic acids and methods

DATE-ISSUED: November 12, 2002

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Rothe; Mike	San Mateo	CA	N/A	N/A
Cao; Zhaodan	Pacifica	CA	N/A	N/A
Regnier; Catherine	South San Francisco	CA	N/A	N/A

APPL-NO: 09/ 109986

DATE FILED: July 2, 1998

PARENT-CASE:

This is a continuing application of U.S. Ser. No. 08/890,854, filed Jul. 10, 1997, which is a continuing application under 35USC120 of U.S. Ser. No. 08/887,115, now abandoned, filed Jul. 1, 1997.

US-CL-CURRENT: 435/194; 435/15

ABSTRACT:

The invention provides methods and compositions relating to an I.kappa.B **kinase, IKK**-.alpha., and related nucleic acids. The polypeptides may be produced recombinantly from transformed host cells from the disclosed **IKK**-.alpha. encoding nucleic acids or purified from **human** cells. The invention provides isolated **IKK**-.alpha. hybridization probes and primers capable of specifically hybridizing with the disclosed **IKK**-.alpha. genes, **IKK**-.alpha.-specific binding agents such as specific antibodies, and methods of making and using the subject compositions in diagnosis, therapy and in the biopharmaceutical industry.

20 Claims, 0 Drawing figures

Exemplary Claim Number: 1

---------- KWIC ---------

Abstract Text - ABTX:

The invention provides methods and compositions relating to an I.kappa.B

kinase, IKK-.alpha., and related nucleic acids. The polypeptides may be produced recombinantly from transformed host cells from the disclosed **IKK**-.alpha. encoding nucleic acids or purified from **human** cells. The invention provides isolated **IKK**-.alpha. hybridization probes and primers capable of specifically hybridizing with the disclosed **IKK**-.alpha. genes, **IKK**-.alpha.-specific binding agents such as specific antibodies, and methods of making and using the subject compositions in diagnosis, therapy and in the biopharmaceutical industry.

Brief Summary Text - BSTX:

Here, we disclose a novel **human kinase** I.kappa.B **Kinase, IKK**-.alpha., as a NIK-interacting protein. **IKK**-.alpha. has sequence similarity to the conceptual translate of a previously identified open reading frame (SEQ ID NO:5) postulated to encode a serine-threonine **kinase** of unknown function (`Conserved Helix-loop-helix Ubiquitous **Kinase**` or CHUK, Connelly and Marcu, 1995; Mock et al., 1995). Catalytically inactive mutants of **IKK**-.alpha. are shown to suppress NF-.kappa.B activation induced by TNF and IL-1 stimulation as well as by TRAF and NIK overexpression; transiently expressed **IKK**-.alpha. is shown to associate with the endogenous I.kappa.B.alpha. complex; and **IKK**-.alpha. is shown to phosphorylate I.kappa.B.alpha. on serines 32 and 36.

Brief Summary Text - BSTX:

The invention provides methods and compositions relating to isolated **IKK**-.alpha. polypeptides, related nucleic acids, polypeptide domains thereof having **IKK**-.alpha.-specific structure and activity and modulators of **IKK**-.alpha. function, particularly I.kappa.B **kinase** activity. **IKK**-.alpha. polypeptides can regulate NF.kappa.B activation and hence provide important regulators of cell function. The polypeptides may be produced recombinantly from transformed host cells from the subject **IKK**-.alpha. polypeptide encoding nucleic acids or purified from mammalian cells. The invention provides isolated **IKK**-.alpha. hybridization probes and primers capable of specifically hybridizing with the disclosed **IKK**-.alpha. gene, **IKK**-.alpha.-specific binding agents such as specific antibodies, and methods of making and using the subject compositions in diagnosis (e.g. genetic hybridization screens for **IKK**-.alpha. transcripts), therapy (e.g. **IKK**-.alpha. **kinase** inhibitors to inhibit TNF signal transduction) and in the biopharmaceutical industry (e.g. as immunogens, reagents for isolating other transcriptional regulators, reagents for screening chemical libraries for lead pharmacological agents, etc.).

Brief Summary Text - BSTX:

The nucleotide sequence of a natural cDNA encoding a **human IKK**-.alpha. polypeptide is shown as SEQ ID NO:3, and the full conceptual translate is shown as SEQ ID NO:4. The **IKK**-.alpha. polypeptides of the invention include incomplete translates of SEQ ID NO:3, particularly of SEQ ID NO:3, residues 1-638, which translates and deletion mutants of SEQ ID NO:4 have human **IKK**-.alpha.-specific amino acid sequence, binding specificity or function and

comprise at least one of Cys30, Leu403, Glu543, Leu604, Thr679, Ser680, Pro684, Thr686 and Ser687. Preferred translates/deletion mutants comprise at least a 6, preferably at least a 12, more preferably at least an 18 residue Cys30, Leu403, Glu543, Leu604, Thr679, Ser680, Pro684, Thr686 or Ser687-containing domain of SEQ ID NO:4, preferably including at least 8, more preferably at least 12, most preferably at least 20 contiguous residues which immediately flank said residue on one, preferably both sides, with said residue preferably residing within said contigous residues, see, e.g. Table 1A, which mutants provide hIKK-.alpha. specific epitopes and immunogens.

Brief Summary Text - BSTX:

The subject domains provide **IKK**-.alpha. domain specific activity or function, such as **IKK**-.alpha.-specific **kinase or kinase** inhibitory activity, NIK-binding or binding inhibitory activity, I.kappa.B-binding or binding inhibitory activity, NF.kappa.B activating or inhibitory activity or antibody binding. Preferred domains phosphorylate at least one and preferably both the serine 32 and 36 of I.kappa.B (Verma, I. M., et al. (1995)). As used herein, Ser32 and Ser36 of I.kappa.B refers collectively to the two serine residues which are part of the consensus sequence DSGL/IXSM/L (e.g. ser 32 and 36 in I.kappa.B.alpha., ser 19 and 23 in I.kappa.B.beta., and ser 157 and 161, or 18 and 22, depending on the usage of methionines, in I.kappa.B.epsilon., respectively.

Brief Summary Text - BSTX:

IKK-.alpha.-specific activity or function may be determined by convenient in vitro, cell-based, or in vivo assays: e.g. in vitro binding assays, cell culture assays, in animals (e.g. gene therapy, transgenics, etc.), etc. Binding assays encompass any assay where the molecular interaction of an IKK-.alpha. polypeptide with a binding target is evaluated. The binding target may be a natural intracellular binding target such as an IKK-.alpha. substrate, a IKK-.alpha. regulating protein or other regulator that directly modulates IKK-.alpha. activity or its localization; or non-natural binding target such a specific immune protein such as an antibody, or an IKK-.alpha. specific agent such as those identified in screening assays such as described below. IKK-.alpha.-binding specificity may assayed by kinase activity or binding equilibrium constants (usually at least about 10.sup.7 M.sup.-1, preferably at least about 10.sup.8 M.sup.-1, more preferably at least about 10.sup.9 M.sup.-1), by the ability of the subject polypeptide to function as negative mutants in IKK-.alpha.-expressing cells, to elicit IKK-.alpha. specific antibody in a heterologous host (e.g. a rodent or rabbit), etc. In any event, the IKK-.alpha. binding specificity of the subject IKK-.alpha. polypeptides necessarily distinguishes the murine and **human CHUK sequences** of Connelly and Marcu (1995) as well as IKK-.beta. (SEQ ID NO:4).

Brief Summary Text - BSTX:

The claimed IKK-.alpha. polypeptides are isolated or pure: an "isolated"

polypeptide is unaccompanied by at least some of the material with which it is associated in its natural state, preferably constituting at least about 0.5%, and more preferably at least about 5% by weight of the total polypeptide in a given sample and a pure polypeptide constitutes at least about 90%, and preferably at least about 99% by weight of the total polypeptide in a given sample. In a particular embodiments, **IKK**-.alpha. polypeptides are isolated from a MKP-1 precipitable complex, isolated from a **IKK** complex, and/or isolated from **IKK**-.beta.. The **IKK**-.alpha. polypeptides and polypeptide domains may be synthesized, produced by recombinant technology, or purified from mammalian, preferably **human** cells. A wide variety of molecular and biochemical methods are available for biochemical synthesis, molecular expression and purification of the subject compositions, see e.g. Molecular Cloning, A Laboratory Manual (Sambrook, et al. Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY) or that are otherwise known in the art.

Brief Summary Text - BSTX:

The invention provides binding agents specific to **IKK** polypeptides, preferably the claimed **IKK**-.alpha. polypeptides, including substrates, agonists, antagonists, natural intracellular binding targets, etc., methods of identifying and making such agents, and their use in diagnosis, therapy and pharmaceutical development. For example, specific binding agents are useful in a variety of diagnostic and therapeutic applications, especially where disease or disease prognosis is associated with improper utilization of a pathway involving the subject proteins, e.g. NF-.kappa.B activation. Novel **IKK**-specific binding agents include **IKK**-specific receptors, such as somatically recombined polypeptide receptors like specific antibodies or T-cell antigen receptors (see, e.g. Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory) and other natural intracellular binding agents identified with assays such as one-, two- and three-hybrid screens, non-natural intracellular binding agents identified in screens of chemical libraries such as described below, etc. Agents of particular interest modulate **IKK** function, e.g. **IKK**-dependent transcriptional activation. For example, a wide variety of inhibitors of **IKK** I.kappa.B **kinase** activity may be used to regulate signal transduction involving I.kappa.B. Exemplary **IKK** I.kappa.B **kinase** inhibitors include known classes of serine/threonine **kinase** (e.g. PKC) inhibitors such as competitive inhibitors of ATP and substrate binding, antibiotics, **IKK**-derived peptide inhibitors, etc., see Tables II and III. **IKK** specificity and activity are readily quantified in high throughput **kinase** assays using panels of protein **kinases** (see cited references and Examples).

Brief Summary Text - BSTX:

Preferred inhibitors include natural compounds such as staurosporine (Omura S, et al. J Antibiot (Tokyo) 1995 July;48(7):535-48), produced by a marine organism, and synthetic compounds such as PD 153035, which also potently inhibits the EGF receptor protein **kinase** (Fry DW et al. Science 1994 August 19;265(5175):1093-5). Members of the tyrphostin family of synthetic protein **kinase** inhibitors are also useful; these include compounds which are pure ATP

competitors, compounds which are pure substrate competitors, and compounds which are mixed competitors: compete with both ATP and substrate (Levitzki A and Gazit A, Science 1995 March 24;267(5205):1782-8). Additional **IKK** inhibitors include peptide-based substrate competitors endogenously made by the mammalian cell, e.g. PKI (protein **kinase** inhibitor, Seasholtz AF et al., Proc Natl Acad Sci USA 1995 February 28;92(5):1734-8), or proteins inhibiting cdc **kinases** (Correa-Bordes J and Nurse P, Cell 1995 December 15;83(6):1001-9). Additional small peptide based substrate competitive **kinase** inhibitors and allosteric inhibitors (inhibitory mechanisms independent of ATP or substrate competition) are readily generated by established methods (Hvalby O, et al. Proc Natl Acad Sci USA 1994 May 24;91(11):4761-5; Barja P, et al., Cell Immunol 1994 January;153(1):28-38; Villar-Palasi C, Biochim Biophys Acta 1994 December 30;1224(3):384-8; Liu W Z, et al., Biochemistry 1994 August 23;33(33):10120-6).

Brief Summary Text - BSTX:

Accordingly, the invention provides methods for modulating signal transduction involving I.kappa.B in a cell comprising the step of modulating **IKK kinase** activity, e.g. by contacting the cell with a serine/threonine **kinase** inhibitor. The cell may reside in culture or in situ, i.e. within the natural host. Preferred inhibitors are orally active in mammalian hosts. For diagnostic uses, the inhibitors or other **IKK** binding agents are frequently labeled, such as with fluorescent, radioactive, chemiluminescent, or other easily detectable molecules, either conjugated directly to the binding agent or conjugated to a probe specific for the binding agent.

Brief Summary Text - BSTX:

The invention provides efficient methods of identifying agents, compounds or lead compounds for agents active at the level of a **IKK** modulatable cellular function. Generally, these screening methods involve assaying for compounds which modulate **IKK** interaction with a natural **IKK** binding target, in particular, **IKK** phosphorylation of I.kappa.B-derived substrates, particularly I.kappa.B and NIK substrates. A wide variety of assays for binding agents are provided including labeled in vitro protein-protein binding assays, immunoassays, cell based assays, etc. The methods are amenable to automated, cost-effective high throughput screening of chemical libraries for lead compounds. Identified reagents find use in the pharmaceutical industries for animal and **human** trials; for example, the reagents may be derivatized and rescreened in in vitro and in vivo assays to optimize activity and minimize toxicity for pharmaceutical development.

Brief Summary Text - BSTX:

In vitro binding assays employ a mixture of components including an **IKK** polypeptide, which may be part of a fusion product with another peptide or polypeptide, e.g. a tag for detection or anchoring, etc. The assay mixtures comprise a natural intracellular IKK binding target. In a particular embodiment, the binding target is a substrate comprising I.kappa.B serines 32

and/or 36. Such substrates comprise a I.kappa.B.alpha., .beta. or .epsilon. peptide including the serine 32 and/or 36 residue and at least 5, preferably at least 10, and more preferably at least 20 naturally occurring immediately flanking residues on each side (e.g. for serine 36 peptides, residues 26-46, 22-42, or 12-32 or 151-171 for I.kappa.B.alpha., .beta. or .epsilon.-derived substrates, respectively). While native full-length binding targets may be used, it is frequently preferred to use portions (e.g. peptides) thereof so long as the portion provides binding affinity and avidity to the subject **IKK** polypeptide conveniently measurable in the assay. The assay mixture also comprises a candidate pharmacological agent. Candidate agents encompass numerous chemical classes, though typically they are organic compounds; preferably small organic compounds and are obtained from a wide variety of sources including libraries of synthetic or natural compounds. A variety of other reagents may also be included in the mixture. These include reagents like ATP or ATP analogs (for **kinase** assays), salts, buffers, neutral proteins, e.g. albumin, detergents, protease inhibitors, nuclease inhibitors, antimicrobial agents, etc. may be used.

Brief Summary Text - BSTX:

After incubation, the agent-biased binding between the **IKK** polypeptide and one or more binding targets is detected by any convenient way. For **IKK kinase** assays, `binding` is generally detected by a change in the phosphorylation of a **IKK**-.alpha. substrate. In this embodiment, **kinase** activity may quantified by the transfer to the substrate of a labeled phosphate, where the label may provide for direct detection as radioactivity, luminescence, optical or electron density, etc. or indirect detection such as an epitope tag, etc. A variety of methods may be used to detect the label depending on the nature of the label and other assay components, e.g. through optical or electron density, radiative emissions, nonradiative energy transfers, etc. or indirectly detected with antibody conjugates, etc.

Brief Summary Paragraph Table - BSTL:

TABLE II Selected Small Molecule **IKK Kinase** Inhibitors HA-100.sup.1 Iso-H7.sup.12 A-3.sup.18 Chelerythrine.sup.2 PKC 19-31 HA1004.sup.19,20 Staurosporine.sup.3,4,5 H-7.sup.12,3,14 K-252a.sup.16,5 Calphostin C.sup.6,7,8,9 H-89.sup.15 KT5823.sup.16 K-252b.sup.10 KT5720.sup.16 ML-9.sup.21 PKC 19-36.sup.11 cAMP-depPKinhib.sup.17 KT5926.sup.22 Citations .sup.1 Hagiwara, M,. et al. Mol. Pharmacol. 32: 7(1987) .sup.2 Herbert, J. M., et al. Biochem Biophys Res Com 172: 993 (990) .sup.3 Schachtele, C., et al. Biochem Biophys Res Com 151: 542 (1988) .sup.4 Tamaoki, T., et al. Biochem Biophys Res Com 135: 397 (1986) .sup.5 Tischler, A. S., et al. J. Neurochemistry 55: 1159 (1990) .sup.6 Bruns, R. F., et al. Biochem Biophys Res Com 176: 288 (1991) .sup.7 Kobayashi, E., et al. Biochem Biophys Res Com 159: 548 (1989) .sup.8 Tamaoki, T., et al Adv2nd Mass Phosphoprotein Res 24: 497 (1990) .sup.9 Tamaoki, T., et al. Biotechnology 8: 732 (1990) .sup.10 Yasuzawa, T. J. Antibiotics 39: 1972 (1986) .sup.11 House, C., et al. Science 238: 1726 (1987) .sup.12 Quick, J., et al. Biochem. Biophys. Res. Com. 167: 657 (1992) .sup.13 Bouli, N. M. and Davis, M. Brain Res. 525: 198 (1990) .sup.14 Takahashi, I., et al. J. Pharmacol. Exp. Ther. 255: 1218 (1990)

.sup.15 Chijiwa, T., et al J. Biol. Chem. 265: 5267 (1990) .sup.16 Kase, H., et al. Biochem. Biophys. Res. Com. 142: 436 (1987) .sup.17 Cheng, H. C., et al. J. Biol. Chem. 261: 989 (1986) .sup.18 Inagaki, M., et al. Mol. Pharmacol. 29: 577 (1986) .sup.19 Asano, T. and Hidaka, H. J Pharmaco. Exp Ther 231: 141 (1984) .sup.20 Hidaka, H., et al. Biochemistry 23: 5036 (1984) .sup.21 Nagatsu, T., et al. Biochem Biophys Res Com 143: 1045 (1987) .sup.22 Nakanishi S., et al. Mol. Pharmacol. 37: 428 (1990)

Brief Summary Paragraph Table - BSTL:

TABLE III Selected Peptidyl **IKK Kinase** Inhibitors hI.kappa.B.alpha., residues 24-39, 32Ala hIKK-.alpha., .DELTA.5-203 hI.kappa.B.alpha., residues 29-47, 36Ala hIKK-.alpha., .DELTA.1-178 hI.kappa.B.alpha., residues 26-46, 32/36Ala hIKK-.alpha., .DELTA.368-756 hI.kappa.B.beta., residues 25-38, 32Ala hIKK-.alpha., .DELTA.460-748 hI.kappa.B.beta., residues 30-41, 36Ala hIKK-.alpha., .DELTA.1-289 hI.kappa.B.alpha., residues 26-46, 32/36Ala hIKK-.alpha., .DELTA.12-219 hI.kappa.B.epsilon., residues 24-40, 32Ala hIKK-.alpha., .DELTA.307-745 hI.kappa.B.epsilon., residues 31-50, 36Ala hIKK-.alpha., .DELTA.319-644 hI.kappa.B.epsilon., residues 27-44, 32/36Ala

Detailed Description Text - DETX:

To investigate the mechanism of NIK-mediated NF-.kappa.B activation, we identified proteins that associate directly with NIK by yeast two-hybrid protein interaction cloning (Fields and Song, 1989). An expression vector was generated that encodes NIK fused to the DNA-binding domain of the yeast transcription factor GAL4. This vector was used as bait in a two-hybrid screen of a **human** B cell cDNA library. From approximately six million transformants, eight positive clones were obtained, as determined by activation of his and lacZ reporter genes. Of these clones, three encoded a member of the TRAF family, TRAF3 (Hu et al., 1994; Cheng et al., 1995; Mosialos et al., 1995; Sato et al., 1995) and one encoded a novel protein we call **IKK**-.alpha.. Retransformation into yeast cells verified the interaction between NIK and **IKK**-.alpha.. A full-length **human IKK**-.alpha. clone was isolated by screening a Jurkat cDNA library with a probe generated from the 5'-end of the **IKK**-.alpha. two-hybrid clone. **IKK**-.alpha. comprises an N-terminal serine-threonine **kinase** catalytic domain, a C-terminal helix-loop-helix domain and a leucine zipper-like amphipathic a-helix juxtaposed in between the helix-loop-helix and **kinase** domain.

Detailed Description Text - DETX:

Interaction of **IKK**-.alpha. and NIK in **Human** Cells

Detailed Description Text - DETX:

The interaction of **IKK**-.alpha. with NIK was confirmed in mammalian cell coimmunoprecipitation assays. **Human IKK**-.alpha. containing an N-terminal Flag

epitope tag was transiently coexpressed in 293 **human** embryonic kidney cells with Myc epitope-tagged NIK or HA epitope-tagged TRAF proteins. Cell lysates were immunoprecipitated using a monoclonal antibody against the Flag epitope, and coprecipitating NIK or TRAF proteins were detected by immunoblot analysis with an anti-Myc or anti-HA monoclonal antibodies. In this assay, **IKK**-.alpha. was able to coprecipitate NIK confirming the interaction between both proteins as detected for **IKK**-.alpha. by yeast two-hybrid analysis. Also, a deletion mutant **IKK**-.alpha. protein lacking most of the N-terminal **kinase** domain (**IKK**-.alpha..sub.(307-745)) was able to associate with NIK, indicating that the .alpha.-helical C-terminal half of **IKK**-.alpha. mediates the interaction with NIK. In contrast to NIK **IKK**-.alpha. failed to associate with either TRAF2 or TRAF3. However, when NIK was coexpressed with **IKK**-.alpha. and TRAF2, strong coprecipitation of TRAF2 with **IKK**-.alpha. was detected, indicating the formation of a ternary complex between **IKK**-.alpha., NIK and TRAF2.

Detailed Description Text - DETX:

We next determined the effect of overexpression of **kinase**-inactive **IKK**-.alpha..sub.(307-745) that still associates with NIK on signal-induced NF-.kappa.B activation in reporter gene assays in 293 cells. Overexpression of **IKK**-.alpha..sub.307-745) blocked TNF- and IL-1-induced reporter gene activation similar to overexpression of NIK.sub.(624-947). **IKK**-.alpha..sub.(307-745) was also found to inhibited NF-.kappa.B-dependent reporter gene activity elicited by overexpression of TRAF2, TRAF6 and NIK. Taken together these results demonstrate that a catalytically inactive **IKK**-.alpha. mutant is a dominant-negative inhibitor of TNF-, IL-1, TRAF- and NIK-induced NF-.kappa.B activation. This indicates that **IKK**-.alpha. functions as a common mediator of NF-.kappa.B activation by TNF and IL-1 downstream of NIK.

Detailed Description Text - DETX:

A. Reagents: Neutralite Avidin: 20 .mu.g/ml in PBS. **kinase:** 10.sup.-8 -10.sup.-5 M **IKK**-.alpha. (SEQ ID NO:4) at 20 .mu.g/ml in PBS. substrate: 10.sup.-7 -10.sup.-4 M biotinylated substrate (21 residue peptide consisting of residues 26-46 of **human** I.kappa.B.alpha.) at 40 .mu.g/ml in PBS. Blocking buffer: 5% BSA, 0.5% Tween 20 in PBS; 1 hour at room temperature. Assay Buffer: 100 mM KCl, 10 mM MgCl.sub.2, 1 mM MnCl.sub.2, 20 mM HEPES pH 7.4, 0.25 mM EDTA, 1% glycerol, 0.5% NP-40, 50 mM BME, 1 mg/ml BSA, cocktail of protease inhibitors. [.sup.32 P].gamma.-ATP 10.times. stock: 2.times.10.sup.-5 M cold ATP with 100 .mu.Ci [.sup.32 P].gamma.-ATP. Place in the 4.degree. C. microfridge during screening. Protease inhibitor cocktail (1000.times.): 10 mg Trypsin Inhibitor (BMB # 109894), 10 mg Aprotinin (BMB # 236624), 25 mg Benzamidine (Sigma # B-6506), 25 mg Leupeptin (BMB # 1017128), 10 mg APMSF (BMB # 917575), and 2 mM NaVo.sub.3 (Sigma # S-6508) in 10 ml of PBS.

Claims Text - CLTX:

20. A method of screening for an agent which modulates the ability of an IKK polypeptide to specifically phosphorylate an I.kappa.B polypeptide, said method

comprising the steps of: incubating a mixture comprising: an isolated polypeptide according to claim 1 retaining I.kappa.B **kinase** activity, an I.kappa.B polypeptide comprising at least a six residue domain of a natural I.kappa.B comprising at least one of Ser32 and Ser36, and a candidate agent; under conditions whereby, but for the presence of said agent, said polypeptide specifically phosphorylates said I.kappa.B polypeptide at at least one of said Ser32 and Ser36 at a reference activity; detecting the polypeptide-induced phosphorylation of said I.kappa.B polypeptide at at least one of said Ser32 and Ser36 to determine an agent-biased activity, wherein a difference between the agent-biased activity and the reference activity indicates that said agent modulates the ability of said polypeptide to specifically phosphorylate an I.kappa.B polypeptide.

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  * * * * * * * * * * * * * * STN  Columbus  * * * * * * * * * * * * * * *

FILE 'HOME' ENTERED AT 15:53:54 ON 24 MAR 2003

=> fil .bec
COST IN U.S. DOLLARS                                    SINCE FILE      TOTAL
                                                           ENTRY    SESSION
FULL ESTIMATED COST                                         0.21       0.21

FILES 'MEDLINE, SCISEARCH, LIFESCI, BIOTECHDS, BIOSIS, EMBASE, HCAPLUS, NTIS,
       ESBIOBASE, BIOTECHNO, WPIDS' ENTERED AT 15:54:02 ON 24 MAR 2003
ALL COPYRIGHTS AND RESTRICTIONS APPLY.  SEE HELP USAGETERMS FOR DETAILS.

11 FILES IN THE FILE LIST

=> s ikk?
FILE 'MEDLINE'
L1         1672 IKK?

FILE 'SCISEARCH'
L2          793 IKK?

FILE 'LIFESCI'
L3          337 IKK?

FILE 'BIOTECHDS'
L4           31 IKK?

FILE 'BIOSIS'
L5          722 IKK?

FILE 'EMBASE'
L6          515 IKK?

FILE 'HCAPLUS'
L7          750 IKK?

FILE 'NTIS'
L8           35 IKK?

FILE 'ESBIOBASE'
L9          462 IKK?

FILE 'BIOTECHNO'
L10         356 IKK?

FILE 'WPIDS'
L11          61 IKK?

TOTAL FOR ALL FILES
L12        5734 IKK?

=> s l12(8a)(human or mouse or murine)
FILE 'MEDLINE'
        8026017 HUMAN
         237075 MOUSE
         102860 MURINE
L13           60 L1 (8A)(HUMAN OR MOUSE OR MURINE)

FILE 'SCISEARCH'
        1012577 HUMAN
         252894 MOUSE
         104489 MURINE
L14           61 L2 (8A)(HUMAN OR MOUSE OR MURINE)
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FILE 'LIFESCI'
        315074 HUMAN
         95037 MOUSE
         45723 MURINE
L15         37 L3  (8A)(HUMAN OR MOUSE OR MURINE)

FILE 'BIOTECHDS'
         51994 HUMAN
         22277 MOUSE
          2170 MURINE
L16         11 L4  (8A)(HUMAN OR MOUSE OR MURINE)

FILE 'BIOSIS'
       5397013 HUMAN
        659442 MOUSE
        134957 MURINE
L17         87 L5  (8A)(HUMAN OR MOUSE OR MURINE)

FILE 'EMBASE'
       4633126 HUMAN
        510313 MOUSE
         91946 MURINE
L18         45 L6  (8A)(HUMAN OR MOUSE OR MURINE)

FILE 'HCAPLUS'
       1107202 HUMAN
        274969 MOUSE
         92193 MURINE
L19        108 L7  (8A)(HUMAN OR MOUSE OR MURINE)

FILE 'NTIS'
         80030 HUMAN
          3893 MOUSE
           895 MURINE
L20           1 L8  (8A)(HUMAN OR MOUSE OR MURINE)

FILE 'ESBIOBASE'
        341084 HUMAN
         81234 MOUSE
         35932 MURINE
L21         44 L9  (8A)(HUMAN OR MOUSE OR MURINE)

FILE 'BIOTECHNO'
        675120 HUMAN
        208395 MOUSE
         52883 MURINE
L22         38 L10(8A)(HUMAN OR MOUSE OR MURINE)

FILE 'WPIDS'
        119310 HUMAN
         18435 MOUSE
          2893 MURINE
L23         10 L11(8A)(HUMAN OR MOUSE OR MURINE)

TOTAL FOR ALL FILES
L24        502 L12(8A)(HUMAN OR MOUSE OR MURINE)

=> s l24 not 2000-2003/py
FILE 'MEDLINE'
       1605574 2000-2003/PY
L25         18 L13 NOT 2000-2003/PY

FILE 'SCISEARCH'
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       3052675 2000-2003/PY
L26          19 L14 NOT 2000-2003/PY

FILE 'LIFESCI'
        306553 2000-2003/PY
L27          16 L15 NOT 2000-2003/PY

FILE 'BIOTECHDS'
         53674 2000-2003/PY
L28           4 L16 NOT 2000-2003/PY

FILE 'BIOSIS'
       1665276 2000-2003/PY
L29          18 L17 NOT 2000-2003/PY

FILE 'EMBASE'
       1367919 2000-2003/PY
L30          17 L18 NOT 2000-2003/PY

FILE 'HCAPLUS'
       3068180 2000-2003/PY
L31          21 L19 NOT 2000-2003/PY

FILE 'NTIS'
         49127 2000-2003/PY
L32           0 L20 NOT 2000-2003/PY

FILE 'ESBIOBASE'
        884529 2000-2003/PY
L33          16 L21 NOT 2000-2003/PY

FILE 'BIOTECHNO'
        364451 2000-2003/PY
L34          15 L22 NOT 2000-2003/PY

FILE 'WPIDS'
       2738049 2000-2003/PY
L35           2 L23 NOT 2000-2003/PY

TOTAL FOR ALL FILES
L36         146 L24 NOT 2000-2003/PY

=> fil .becpat
COST IN U.S. DOLLARS                                SINCE FILE      TOTAL
                                                       ENTRY    SESSION
FULL ESTIMATED COST                                    15.27      15.48

FILES 'BIOTECHDS, HCAPLUS, WPIDS' ENTERED AT 15:55:50 ON 24 MAR 2003
ALL COPYRIGHTS AND RESTRICTIONS APPLY.  SEE HELP USAGETERMS FOR DETAILS.

3 FILES IN THE FILE LIST

=> s l24 and wo/pc and pry<=1999 and py>=2001 range=2000,
FILE 'BIOTECHDS'
          20388 WO/PC
          17358 PRY<=1999
                (PRY<=1999)
          38256 PY>=2001
                (PY>=2001)
L37           0 L16 AND WO/PC AND PRY<=1999 AND PY>=2001

FILE 'HCAPLUS'
         139040 WO/PC
         352699 PRY<=1999
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       2020939 PY>=2001
L38          4 L19 AND WO/PC AND PRY<=1999 AND PY>=2001

FILE 'WPIDS'
        297333 WO/PC
       1328942 PRY<=1999
                 (PRY<=1999)
       1581729 PY>=2001
                 (PY>=2001)
L39          0 L23 AND WO/PC AND PRY<=1999 AND PY>=2001

TOTAL FOR ALL FILES
L40          4 L24 AND WO/PC AND PRY<=1999 AND PY>=2001

=> d tot

L40  ANSWER 1 OF 4  HCAPLUS  COPYRIGHT 2003 ACS
TI   Purification and characterization of human stimulus-inducible
     I.kappa.B kinase (IKK) signalsome and possible use of IKK for
     the treatment of diseases associated with NF-.kappa.B activation
SO   U.S., 58 pp., Cont.-in-part of U.S. 5,972,674.
     CODEN: USXXAM
IN   Mercurio, Frank; Zhu, Hengyi; Barbosa, Miguel; Li, Jian Wu; Murray, Brion
     W.
AN   2001:499792  HCAPLUS
DN   135:104267
     PATENT NO.        KIND  DATE          APPLICATION NO.  DATE
     ---------------   ----  --------      ---------------  --------
PI   US 6258579         B1   20010710      US 1997-910820   19970813 <--
     US 5972674         A    19991026      US 1996-697393   19960826
     WO 9808955         A1   19980305      WO 1997-US15003  19970826 <--
         W:  AU, CA, JP
         RW: AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE
     AU 9740904         A1   19980319      AU 1997-40904    19970826 <--
     AU 726383          B2   20001102
     EP 920518          A1   19990609      EP 1997-938616   19970826 <--
         R:  AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT,
             IE, FI
     JP 2001502892      T2   20010306      JP 1998-511840   19970826 <--
     US 2002151021      A1   20021017      US 2001-844908   20010427 <--

L40  ANSWER 2 OF 4  HCAPLUS  COPYRIGHT 2003 ACS
TI   Cloning, sequencing and characterization of human IKK4
     kinase and use of the IKK4 in screening for anti-inflammatory
     agents
SO   PCT Int. Appl., 31 pp.
     CODEN: PIXXD2
IN   Hashimoto, Yasuhiro; Takemoto, Yoshihiro; Furuta, Masaaki; Sakai, Yutaka
AN   2001:453233  HCAPLUS
DN   135:57859
     PATENT NO.        KIND  DATE          APPLICATION NO.  DATE
     ---------------   ----  --------      ---------------  --------
PI   WO 2001044444      A2   20010621      WO 2000-JP8873   20001214 <--
     WO 2001044444      A3   20020510
         W:  AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN,
             CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR,
             HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT,
             LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU,
             SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN,
             YU, ZA, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM
         RW: GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW, AT, BE, CH, CY,
             DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR, BF,
             BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG
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L40 ANSWER 3 OF 4 HCAPLUS COPYRIGHT 2003 ACS
TI Method based on IKK-.beta. interaction for identifying compounds for treatment of insulin resistance
SO PCT Int. Appl., 13 pp.
CODEN: PIXXD2
IN Shoelson, Steven
AN 2001:114936 HCAPLUS
DN 134:141753

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	WO 2001010384	A2	20010215	WO 2000-US21805	20000810 <--
	WO 2001010384	A3	20010607		

W: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM
RW: GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW, AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG

	AU 2000068987	A5	20010305	AU 2000-68987	20000810 <--

L40 ANSWER 4 OF 4 HCAPLUS COPYRIGHT 2003 ACS
TI **Human** I.kappa.B kinase **IKK3**, cDNA coding for it, and uses in screening anti-inflammatory agents
SO PCT Int. Appl., 102 pp.
CODEN: PIXXD2
IN Takemoto, Yoshihiro; Sakai, Yutaka; Hashimoto, Yasuhiro
AN 2000:457220 HCAPLUS
DN 133:86094

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	WO 2000039308	A1	20000706	WO 1999-JP7286	19991224 <--

W: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM
RW: GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW, AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG

	EP 1141325	A1	20011010	EP 1999-961374	19991224 <--

R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT, IE, SI, LT, LV, FI, RO

	JP 2002533127	T2	20021008	JP 2000-591199	19991224 <--

=> log y
COST IN U.S. DOLLARS

	SINCE FILE ENTRY	TOTAL SESSION
FULL ESTIMATED COST	28.60	44.08

STN INTERNATIONAL LOGOFF AT 15:59:54 ON 24 MAR 2003